PROGRESS IN MEDICINAL CHEMISTRY 21

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Preface

Six reviews are presented in this volume. Chapter 1 surveys the wide variety of compounds which have recently been shown in laboratory tests to have antiallergic action. Considerable effort has been generated in the search for therapeutic compounds based on sodium cromoglycate, but knowledge is still incomplete on its mode of action. Drug therapy in this field has greatly improved over the past 20 years and, as compounds experimentally more than 2000-times as active as cromoglycate have recently been synthesized, we can look forward to further improvement at the clinical level in the future.

Problems encountered in classifying snakes into families and their venoms into pharmacological actions on nerve and muscle form the basis of Chapter 2. The importance of the purity of these polypeptide products is stressed. Further work is clearly needed in this field, especially in standardization of procedures.

Interest in indazole carboxylic acids was raised by the observation that some of these possess an antispermatogenic activity. During a study of their mode of action, some were found also to possess antitumour activity, both germ and cancer cell respiration being inhibited. Chapter 3 discusses this group of compounds and focusses on the therapeutic activity, for example, of lonidamine in cancer therapy and tolnidamine as a male contraceptive.

Many of the clinical problems of present-day therapy of Parkinson's disease derive from centrally induced adverse reactions to continuing large doses of dihydroxyphenylalanine. Chapter 4 describes how potentiation of the amino acid action can be achieved with lower dosage (and thereby fewer adverse effects) by using potent inhibitors of the enzyme responsible for deamination of dopamine formed by the decarboxylation of dihydroxyphenylalanine. In particular, the evolution of one such inhibitor, called selegiline, is traced in detail.

New potentially clinically useful analogues of doxorubicin, an antibiotic antitumour drug, have been identified by screening natural and semisynthetic products. These anthracyclines form a very valuable series of compounds and are reviewed in Chapter 5. Cardiotoxicity and antitumour effects have at last been separated and the full potential of such compounds in cancer therapy remains to be discovered in the near future.

The discovery that blood vessels synthesize and release an unstable

arachidonic acid metabolite called prostacyclin has led to the suggestion that this compound may play an important factor in the regulation of normal vascular bone and haemostasis. Being a potent vasodilator and inhibitor of platelet aggregation, prostacyclin may thus have many clinical applications in the management of vascular disorders. This compound and many of its analogues are assessed in Chapter 6 and shown to be capable of selective action on blood vessels and platelets.

Our authors are experts in their fields and we are grateful to them for the time and effort they have devoted to writing these reviews. We are grateful also to the owners of copyright material which we have included. Finally, as always, we are indebted to the staff of our publishers for their full co-operation.

G.P. Ellis
February 1984 G.B. West

Contents

Progress in Medicinal Chemistry – Vol. 21, edited by G.P. Ellis and G.B. West

1 The Search for Antiallergic Drugs for the Treatment of Asthma – Problems in Finding a Successor to Sodium Cromoglycate

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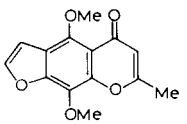
INTRODUCTION – DISCOVERY OF SODIUM CROMOGLYCATE

The naturally occurring antispasmodic khellin (1) [1], a furanochromone, formed the basis of a project initiated at Benger Laboratories (Holmes Chapel, Cheshire, U.K.) in the mid-1950's to develop a compound with improved bronchodilator potency and solubility, and which was devoid of the side-effects (nausea, vomiting) associated with khellin itself. Derivatives (2) were produced amongst which were some in which these conditions were in part met, but for various reasons none was developed as a marketable drug. However, the



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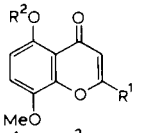

R^1 and R^2 = alkyl

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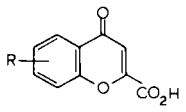

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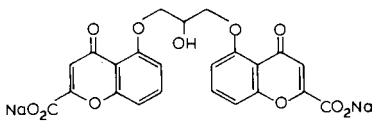
project took a critical shift in direction by the involvement of an asthmatic physician, Dr. Roger Altounyan, who evaluated new compounds by inhalation in himself [2]. He made the shrewd observation that khellin derivatives (3) in which the 2-methyl group had been replaced by a carboxylic acid function, though possessing no bronchodilator properties, prophylactically inhibited the bronchoconstriction induced by an antigen to which he was sensitive. Thus a fixed dose of the chromone-2-carboxylate was inhaled as a nebulized aqueous solution of the sodium salt at standard times before antigen challenge. The impairment of lung function (forced expiratory volume in 1 s, FEV_1) was compared with control values and expressed as a percentage protection for each new compound. It was through the use of this test that the newly found 'antiallergic' or 'antiasthmatic' activity was optimized, and its development culminated in the discovery of sodium cromoglycate (4; Intal®) [3], which was first marketed in the U.K. in 1968. The medicinal chemistry of analogues has been described [4].



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Throughout this text, the term antiallergic will be used to refer to compounds which, when given prophylactically, inhibit certain Type I immediate hypersensitivity reactions caused by the interaction of antigen with cell-fixed immunoglobulin E (IgE), and which are clinically expressed in such diseases as allergic asthma, seasonal and perennial rhinitis and allergic conjunctivitis. Such antiallergic compounds would be expected, except where indicated, to have a mode of action similar to that of sodium cromoglycate.

THE MODE OF ACTION OF SODIUM CROMOGLYCATE

The primary mode of action of sodium cromoglycate is believed to be that of stabilizing mast cells and thereby preventing the subsequent secretion of mediators induced by antigen and other secretagogues. The release of mediators from other cells, for example, histamine release from human basophil leucocytes [5] and SRS-A release from rat polymorphonuclear leucocytes (neutrophils) [6], is generally not inhibited by sodium cromoglycate, although recently beta-glucuronidase release from human alveolar macrophages has been claimed to be inhibited by the drug [7].

In common with other secretory systems, the antigen-induced release of histamine and other mediators from mast cells requires extracellular calcium. Sodium cromoglycate has been shown to inhibit the uptake of radioactive calcium into rat purified mast cells following stimulation with compound 48/80 [8, 9] or antigen [10]. A number of observations suggest that the effect of the drug is exerted at or near the mast-cell surface. For example, there is an alteration in the surface electrokinetic properties of unstimulated rat mast cells in the presence of sodium cromoglycate, as demonstrated by their increased electrophoretic mobility [9]. Also, it has been shown that sodium cromoglycate inhibits the clustering of glycosphingolipids, possibly gangliosides located in the outer leaflet of the plasma membrane bilayer of rat mast cells [11]. Additionally, sodium cromoglycate, covalently conjugated to fluorescent polyacrylamide or polyglutaraldehyde beads, has been shown to bind to the membrane of rat mast cells [12].

The most attractive biochemical basis for an explanation of the mode of action of sodium cromoglycate derives from studies which have shown that the compound induces phosphorylation of a protein of molecular weight 78,000 in purified unstimulated rat peritoneal mast cells [13, 14] at concentrations in keeping with those which inhibit histamine secretion induced by compound 48/80 [13] or anti-IgE [14]. It was suggested that phosphorylation of this particular protein, which also occurs 30–60 s after stimulation of these cells

with compound 48/80 [15] or anti-IgE [14], was associated with turning off the secretory response [13]. Thus, sodium cromoglycate has been shown to activate a putative natural secretion control mechanism.

Protein phosphorylations are known to be catalysed by protein kinases. Phosphorylation of the 78,000 molecular weight protein was also shown to be induced by dibutyryl cyclic GMP but not by dibutyryl cyclic AMP, suggesting that it is mediated through the action of a cyclic-GMP-dependent protein kinase [14].

Although sodium cromoglycate has been reported to be an inhibitor of cyclic-GMP phosphodiesterase [16, 17] (and of cyclic-AMP phosphodiesterase [16, 18]) in a variety of cell-free systems, at high concentrations, it is unlikely that such a hydrophilic compound with a high acidity (pK_1 1.1) attains the intracellular levels necessary for phosphodiesterase inhibition. Wells and Mann [14] have therefore suggested that a more logical alternative for the action of sodium cromoglycate would be stimulation of guanylate cyclase. This enzyme has both soluble and membrane-bound forms and thus this postulate fits well with the idea of the drug acting at the level of the mast-cell plasma membrane to prevent the entry of calcium into the cell. In this respect it is interesting that the cromoglycate-like antiallergy drug, lodoxamide (see p. 41), at high concentrations has been reported to stimulate preferentially the guanylate cyclase of rat lung [19].

Sodium cromoglycate may thus activate a natural control mechanism to terminate mediator release and therefore interrupt the whole sequence of events which results in the symptoms of immediate hypersensitivity reactions such as bronchial asthma, and which leads to inflammatory changes and cell damage. A consequence of this would be an amelioration of the mediator-induced increase in bronchial mucosal permeability (i.e., opening of tight junctions in the epithelium of the airways [20]), which would limit the access of allergens and nonspecific stimuli to sub-epithelial mast and other cells (for example, nerve and respiratory smooth muscle cells). Thus, sodium cromoglycate has the capability of reducing the hyperreactivity of the airways which is such a characteristic feature of asthma. This may, in fact, be the most important therapeutic effect of the drug [21].

Sodium cromoglycate has also been shown to inhibit bronchoconstriction induced in man by a variety of non-immunological triggers, for example, hyperventilation due to exercise [22], voluntary hyperventilation [23], and inhalation of cold air [24], sulphur dioxide [25] or ultrasonically nebulized distilled water ('fog') [26]. It is likely that vagal reflex mechanisms are important in some or all of these responses, although mediator release from mast cells may be involved in the bronchoconstrictor response to exercise [27] and 'fog' [28], in

particular. Thus it appears that not all of the actions of sodium cromoglycate can be explained on the basis of mast-cell stabilization. A non-mast-cell effect of the drug which has been demonstrated in animals is that of inhibition of histamine aerosol-induced airway constriction of vagal reflex origin in the dog [29]. This was shown not to be due to an effect of sodium cromoglycate on lung irritant receptors, as had been proposed [30]. However, the drug was subsequently shown to inhibit excitatory responses of 'C' fibre sensory nerve endings in the dog lung [31]. Stimulation of these particular pulmonary nerve endings in the dog is believed to initiate reflex bronchoconstrictor responses [32]. If similar sensory nerves exist in man and their activation results in bronchoconstriction, then this neuropharmacological action of sodium cromoglycate may explain its inhibitory effect on reflex-induced bronchoconstriction in man. In the guinea-pig also, sodium cromoglycate has been shown to produce inhibition of a component of the bronchoconstriction induced by leukotriene D_4 which is mediated by a vagal reflex [33].

Sodium cromoglycate has also recently been shown to inhibit platelet-activating factor (PAF-acether)-induced inflammatory responses in the skin of guinea-pig and man, and it has therefore been suggested that this is an alternative mechanism of action of the drug in asthma [34]. However, the drug does not inhibit allergic reactions in the skin of man [35], and moreover it has now been shown that (given by aerosol or i.v.) it does not inhibit PAF-acether-induced bronchoconstrictor responses in guinea-pigs [36]. It is unlikely, therefore, that the mechanism of the antiallergic effect of sodium cromoglycate is dependent on inhibition of PAF. The drug also does not antagonize the actions of other putative mediators of allergic reactions, for example, histamine, leukotrienes, bradykinin, 5-hydroxytryptamine, acetylcholine [3], except at concentrations (10^{-2} M vs. histamine on guinea-pig ileum) [37] which are most unlikely ever to be achieved in the vicinity of end-organ receptors in the airways of man [38].

Although acute bronchodilator effects have been reported following sodium cromoglycate administration in man (e.g., Refs. 39, 40), other groups have disputed this (e.g., Refs. 41, 42) and no evidence for a bronchodilator effect of the compound was seen in animal pharmacology experiments *in vitro* or *in vivo* [3].

PHARMACOLOGICAL AND CLINICAL EVALUATION OF ANTIALLERGIC COMPOUNDS

Although sodium cromoglycate was discovered by experiments in man, other animal or *in vitro* tests have since been developed for use in screening

compounds for sodium cromoglycate-like activity. The laboratory rat has provided many of these test systems and, in particular, the passive cutaneous anaphylaxis (PCA) test [43]. For the reader who may not be familiar with this test, the methodology is worthy of brief note. Rat skin sites are passively sensitized by the intradermal injection of IgE-rich antiserum from other rats and 48–72 h later the recipient rats are challenged with an intravenous injection of specific antigen together with a blue dye. Disruption of mast cells at the passively sensitized skin sites, following antigen-antibody (IgE) interaction, results in the release of chemical substances (mediators) which cause an increase in capillary permeability, delineated by extravasation of blue dye. Administration of sodium cromoglycate with or just prior to antigen causes a reduction in the size of these blueing reactions in the rat skin and the effect of a test compound can be compared with sodium cromoglycate in groups of rats.

Although 16 years have elapsed since the introduction of sodium cromoglycate into clinical practice, in spite of considerable effort by many companies, no successor antiallergic drug has yet been marketed. It is interesting to speculate why this should be so. Problems of drug discovery in this area probably arise from the absence of suitable animal models, the dubious relationship between human challenge experiments and clinical efficacy, and difficulties in the therapeutic evaluation of antiallergic compounds.

First of all, the lack of predictivity of animal models of allergy (in contrast to screens for evaluation of bronchodilator activity) has certainly played a major role in hindering the development of such agents. The almost exclusive use of the rat PCA test by most groups to optimize activity has probably been counterproductive, since this screen gives only a semi-quantitative appreciation of likely antiallergic activity in man [44]. In addition, compounds with differing pharmacological properties are active in rat PCA [45] and consequently the phenomena of tachyphylaxis (self-inhibition) and cross-tachyphylaxis (cross-inhibition between sodium cromoglycate and other compounds) in the rat have been used by many groups to indicate whether these other compounds have a mode of action similar to that of sodium cromoglycate. Also useful in this way is induction of phosphorylation of a 78,000 molecular weight protein in the rat peritoneal mast-cell membrane [14] which has been referred to in the previous section, but which again does not appear to give a quantitative assessment of potential activity in man [46]. Other rat models such as passive lung anaphylaxis *in vivo* [47] or inhibition of mediator release from peritoneal mast cells *in vitro* [46] appear to do no more than reflect activity in rat PCA, particularly when the different bioavailabilities in these systems are taken into consideration. The mouse is unsuitable for determining antiallergic activity, since IgE-mediated reactions in this species are insensitive to the effect of sodium cromoglycate [3].

Similarly, IgG-mediated reactions in the guinea-pig are of no use in screening for sodium cromoglycate-like activity [3]. However, IgE-mediated reactions in the guinea-pig have been reported to be inhibitable by sodium cromoglycate [48–50] and although such reactions have not been used in screening programmes, they could be of use in the preclinical profiling of the activity of new compounds.

Subhuman primates may provide more predictive animal models, but using such animals for routine screening is normally not practicable. *Ascaris*-induced respiratory changes in wild-caught naturally sensitive rhesus monkeys with airway hyperreactivity [51] have been shown to be inhibited by sodium cromoglycate given i.v. or by aerosol [52, 53]. In this same model, lodoxamide, later found to be effective in human antigen provocation experiments, was a more potent inhibitor when given by either the i.v., intrabronchial or oral routes [53]. The reproducibility of lung function changes following repeated antigen challenge can be a problem using this model and, in our hands, the reproducibility of the effect of sodium cromoglycate as a standard compound is not good [46]. In addition, such wild-caught monkeys are not now readily available as experimental animals.

Laboratory-bred stump-tailed macaques, experimentally infected with *Ascaris suum*, have also been used in our laboratories [54]. Antigen-induced changes in respiratory mechanics in these animals were not inhibited by sodium cromoglycate (i.v. infusion or aerosol), but some inhibition was seen with a more potent antiallergic compound, FPL 58668 disodium salt–probicromil (see p. 16) [55]. This mirrors findings in *Ascaris*-sensitive dogs [56]. The airways of these animals were not hyperreactive (to histamine) and consequently a relatively high antigen challenge dose was required to elicit a significant change in airway mechanics. This may, at least in part, explain their refractoriness to sodium cromoglycate.

The effect of drugs on sensitized human lung fragments has been extensively investigated and logically should be the most relevant *in vitro* test for antiallergic activity. Eleven analogues of sodium cromoglycate were studied for their ability to suppress histamine release induced by anti-IgE from passively sensitized human lung fragments. The compounds were all more potent than sodium cromoglycate (30–1500-times), yet several of them have been shown to be inferior in the treatment of asthma [57]. Human lung and rat PCA results also did not correlate.

Sodium cromoglycate induces a species-specific vagally mediated hypotensive response (Bezold-Jarisch-like effect) following intravenous injection in the dog. This effect is due to activation of receptors in the left ventricle of the heart [30] and can be blocked by prior infusion of a low dose of sodium

cromoglycate (or other antiallergic compound). The degree and duration of the blockade in such infusion experiments give an indication of the affinity of a compound for, and its elimination from, these sensory receptors in the dog heart [44]. Receptor-fitting studies such as these can be used, in conjunction with some or all of the tests referred to earlier, to help in building up a profile of sodium cromoglycate-like activity of potential new antiallergy compounds. As used in our laboratories, such hypotension blockade experiments in pentobarbitone-anaesthetized dogs consist, briefly, of giving bolus i.v. doses of sodium cromoglycate (5–25 μg) at 30-min intervals (to avoid tachyphylaxis) until a consistent hypotensive response of about 20–30 ($\pm$ 3) mmHg is obtained. The test compound is then administered as an i.v. infusion of 10–200 μg/kg per min for 15 min. Immediately thereafter, and at further 30-min intervals, the pre-infusion bolus i.v. dose of sodium cromoglycate is repeated for a maximum of 3 h. Percentage inhibition of the hypotensive response is recorded following each injection of sodium cromoglycate and the overall inhibition over 3 h is determined from the area under the curve (AUC) of percentage inhibition against time. This test will be referred to subsequently as the dog hypotension screen. Even when a putative antiallergic agent is deemed to be suitable for testing in man, a major hurdle still has to be negotiated. Before therapeutic trials can be performed, the drug must be evaluated in a bronchial provocation test and the predictivity of these acute tests for the chronic disease is by no means established. The provocation test which is generally thought to be the most relevant for the evaluation of antiallergic compounds is that involving bronchial antigen challenge, the method used originally by Dr. Altounyan and which led to the discovery of sodium cromoglycate. Several compounds which were evaluated in this test and found to be active have subsequently been shown to possess only modest therapeutic efficacy [58]. These compounds are discussed in the appropriate sections later in this review. Two factors in antigen challenge studies have often not been fully considered. Firstly, the importance of duration of action of compounds (i.e., the maximum time between administration of the drug and antigen, which still results in a significant protection being afforded by the drug) is often not investigated. This may have been a reason for the discrepancy in the case of bufrolin (see p. 34) between antigen challenge data and clinical efficacy. Secondly, the ability of compounds to inhibit *late*-onset airway responses to antigen have often not been assessed and may be a much more relevant pointer to clinical efficacy. The failure of doxantrazole (see p. 28) to reach the market-place may have been, in part, a consequence of its inability to attenuate the late reaction in asthmatic patients after antigen challenge.

The main disadvantages of such antigen provocation experiments are the

danger in symptomatic asthmatics associated with antigen-induced bronchoconstriction sufficient to give the commonly used 20% fall in forced expiratory volume in 1 s (FEV_1), and also the risk of late-onset bronchoconstriction responses, particularly following the higher doses of antigen required to produce the same fall in FEV_1 in asymptomatic atopics.

To overcome these problems, partial expiratory flow volume manœuvres are now used in our laboratories to determine partial expiratory flow rate (pEFR), the change in which is more sensitive than FEV_1 as an index of the airway response. This technique enables a minimal antigen challenge to be used, with less hazard to the human volunteers [59].

A number of nonspecific provocation tests, for example, exercise, hyperventilation, inhalation of cold air, ultrasonically nebulized distilled water ('fog') or sulphur dioxide, have also been used for the pretherapeutic testing of potential antiallergy compounds. These tests are relatively easy to perform and less hazardous than normal (i.e., 20% fall in FEV_1) antigen provocation tests. Thus these tests are useful as replacements for, or, more particularly, as supplements to, antigen provocation tests, and are helpful in evaluating the spectrum of activity of compounds. The mechanisms involved in these alternative provocation tests have been alluded to earlier (see p. 4).

There are many problems associated with therapeutic trials of antiallergics in asthma. First of all, the very variable nature of the disease leads to difficulties in the selection of appropriate patients in whom to assess the value of a new compound [60, 61]. Patients are often not sufficiently well instructed in the concept of prophylatic use of such compounds and, in the case of compounds for inhalation, in the correct use of inhalation devices. In addition, a high placebo effect is sometimes observed, particularly in patients with a significant emotional component in their disease [62].

Much of the research into antiallergic agents has been directed towards the identification of compounds which may be taken by mouth. The requirements for activity and for oral absorption have in general been mutually exclusive, since activity is usually associated with highly acidic molecules which are not likely to be absorbed. Some attempts at latentiation of active structures (usually by esterification or other modification of the acid function) have led to improved absorption, but once the parent acid has been liberated in the plasma it may be eliminated rapidly due once again to its low pK_a. This may at least in part account for the poor activity exhibited by some antiallergic agents, even when they are well absorbed.

In the final analysis, however, it is not until successful therapeutic trials have been performed that a new antiallergic (anti-asthma) drug can truly be said to have been discovered.

Sodium cromoglycate has also proved effective in other diseases, for example, seasonal allergic [63] and perennial [64] rhinitis, allergic conjunctivitis [65], vernal keratoconjunctivitis [66], food allergy [67], systemic mastocytosis [68]; reports of its effectiveness in the treatment of mouth ulcers [69] and proctocolitis [70, 71] have also appeared. Of these areas, early clinical evaluation using an acute challenge methodology has been possible only for allergic rhinitis using nasal antigen challenge [72]. Though sodium cromoglycate given orally has been very successful in the treatment of the skin manifestations of food allergy and there are reports of its effectiveness, when topically applied, in childhood eczema, its role in the management of allergic skin disease is yet to be clearly established.

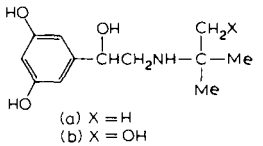
A SURVEY OF STRUCTURES WHICH POSSESS ANTIALLERGIC ACTIVITY

More than fifty pharmaceutical companies have been involved over the last 14 years in the search for new antiallergic drugs. Since sodium cromoglycate is not orally absorbed and is therapeutically effective in asthma only when it is inhaled, the main effort in this area was initially directed towards the development of antiallergic agents which could be taken by mouth. In recent years, however, the identification of inhalation compounds has again been deemed desirable, partly because of the difficulty experienced in developing a satisfactory oral compound but also because of increasing recognition of the advantages of topical treatment [73].

Most of the published efforts to produce cromoglycate-like antiallergic agents are presented in the following sections. Attempts to identify structure-activity relationships are made and the ability of pharmacophores other than chromone-2-carboxylic acid to display such activity will also be discussed. The patent literature (reviewed competently elsewhere [74]) will be largely ignored, since there is very little indication in these publications where biological activity lies or how it can be optimized. Also, due to the great diversity of chemical structures covered, synthetic aspects are generally not covered, though references to medicinal chemical journals are given which normally describe the organic chemistry of the compounds. Where clinical results have been reported these are alluded to, but only a small number of compounds have progressed thus far. Human studies have also been reviewed elsewhere [75].

DISPUTED ANTIALLERGIC AGENTS

The vast majority (if not all) of antiallergic agents with cromoglycate-like activity are acidic compounds (or latentiated derivatives thereof). However, controversy exists over whether β_2-adrenoceptor stimulants possess such activity in addition to their direct bronchodilator action. The ability of terbutaline (5a) and sodium cromoglycate to inhibit mediator release in man has been measured [76, 77]. Both drugs partially inhibited increases in plasma



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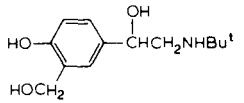

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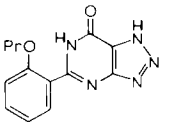
histamine and neutrophil chemotactic activity (NCA) caused by antigen challenge, but the non-β-stimulants theophylline and prednisone behaved similarly. It has been shown [14] that salbutamol (6) does not induce phosphorylation of the 78,000 dalton protein of the rat peritoneal mast cell (see p. 3). Whether or not β-stimulants inhibit histamine release from the same cell is, however, not clearly established. Workers at Johns Hopkins University [78] claimed that there is an inhibitory effect on histamine release, whereas workers at Glaxo-Allenbury's showed it to be minimal [79]. Salbutamol is, however, a potent inhibitor of mediator release from sensitized human lung fragments [79,80].

A close analogue of terbutaline (5b; KWD 2131; Draco) was shown to be equipotent to terbutaline with regard to inhibition of anaphylactic histamine release in the guinea-pig lung, but much less potent as a bronchodilator [81]. KWD 2131 was also a potent inhibitor of histamine release from sensitized human lung fragments. The compound was, therefore, considered to be a probe for the investigation of β-receptor-mediated antiallergic effects in man. Unfortunately, KWD 2131 had no protective effect on lung function changes following antigen challenge in twelve allergic asthmatics [82]. It therefore appears that β-mediated antiallergic effects in animal models are not translated into clinical activity.

The azaxanthine derivative (7; M&B-22948; zaprinast) [83, 84], probably manifests its antiasthmatic activity mainly through phosphodiesterase inhibition, but does also appear to possess antiallergic properties. It is much more potent than theophylline in the rat PCA test [85] and, unlike this compound, has been shown [46] to induce phosphorylation of the 78,000 dalton protein

of the rat peritoneal mast cell (see p. 3). The compound has also been shown to inhibit exercise asthma in the clinic [86, 87], and has been claimed to inhibit antigen-induced bronchospasm in asthmatics [83]. The medicinal chemistry of analogues has been described [88, 89]. Other references to the antiallergic activity of purine analogues are not covered by this review.



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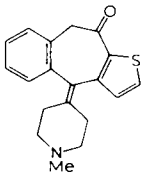

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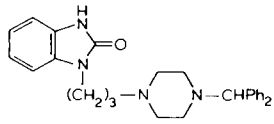
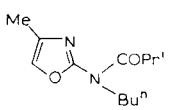


(9)

Ketotifen (8; Sandoz) [90], which is now marketed as a treatment for asthma [91], is primarily a histamine H_1-receptor antagonist which has also been shown in animal studies to have antiallergic activity [90, 92]. It also selectively inhibited the release of SRS-A from human chopped lung [93]. Varying reports of its clinical effectiveness have been published [91]. Further studies are required to establish which of its properties are responsible for its activity in man. Oxatomide (9; Janssen) [94] falls into a category similar to ketotifen.

Isamoxole (10; Lilly), which may be a lipoxygenase inhibitor, shows a preferential ability to inhibit SRS-A release from passively sensitized human chopped lung (like ketotifen), causes human bronchial muscle to relax, and antagonizes the bronchoconstriction induced by SRS-A [93, 95]. Tranilast



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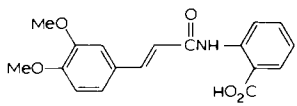
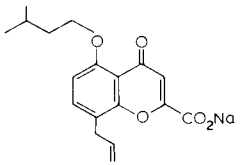


(11)

(11; N5′; Kissei), which has been shown to be therapeutically effective in asthma [96, 97], is now marketed in Japan. Though the compound displays an ability to inhibit the effects of antigen in a number of animal models [98, 99], it is relatively weakly active. In our hands, it was inactive in the dog hypotension screen [46]. In addition, it has other activities, in particular anti-inflammatory, not possessed by sodium cromoglycate.

CHROMONES

The early efforts at finding an orally effective sodium cromoglycate derivative in our laboratories were based on the rat PCA test as a primary screen. Compounds were administered intravenously (i.v.) or intraduodenally (i.duo.). The i.v. result (ID_{50} value i.v.) gave intrinsic potency, whereas the ratio of the values ID_{50}i.duo./ID_{50}i.v. was an index of oral absorption. Structure-activity relationships in a series of 5-alkoxychromone-2-carboxylates have already been described [44]. Activity was maximized for the 5-isopentyloxy (5-isoamyloxy) derivative (12; FPL 55618), which was 87-times more potent than sodium cromoglycate by the i.v. route. This compound was taken to the clinic but was



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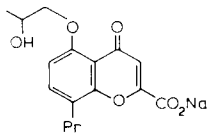


(13)

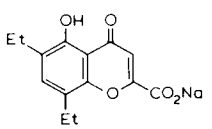
found to have only minimal activity against human antigen challenge [44]. Nevertheless, when given orally to man, FPL 55618 was capable of blocking the protective effect afforded by inhaled sodium cromoglycate against bronchoconstriction induced by antigen. This suggested at the time that the rat PCA test was perhaps capable of measuring a 'fit' on the human sodium cromoglycate receptor, but that it would not predict efficacy. Another compound, which showed moderate activity in the rat PCA test (twice as potent as sodium cromoglycate by the i.v. route) was the structure (13; 5-(2-hydroxypropoxy)-8-propylchromone-2-carboxylate; FPL 52694) which, unlike the isopentyloxy compound (12), was effective orally in man against bronchial antigen challenge, albeit at high doses (1–2 g). The lack of potency, due at least in part to a poor absorption profile, resulted in a halting of its development for the treatment of asthma, but the compound has more recently been shown to inhibit gastric acid secretion in animals [100] and man [101] and may be useful in the treatment of peptic ulcers. This newly found activity may also be a consequence of its mast-cell-stabilizing properties.

The poor absorption of compounds (12) and (13) is largely predictable from their low lipophilicities (log $D^{*}_{7.4}$ = 0.61 and − 0.86, respectively) and also from

* $D_{7.4}$ is the octanol-water distribution coefficient at pH 7.4.

their high acid strength ($pK_a \approx 1.6$); the latter property ensures that the compounds are almost completely ionized in the gastrointestinal tract at all but the pH of the stomach.

The search for more potent, better absorbed chromone-2-carboxylic acids was aided by the observation that the introduction of a 5-hydroxy function was both beneficial to activity and increased the lipophilicity of the molecule ($\pi_{5\text{-OH}} \approx 0.6$). At the same time, the dog hypotension screen (see p. 8) was adopted by us as a new primary screen. The 5-hydroxy-6,8-diethyl compound (14; FPL 52757) [102] was identified and taken forward for clinical studies.



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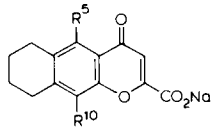


(15) $R^5 = R^{10} = H$
(16) $R^5 = OH$; $R^{10} = Pr$

The compound was withdrawn when hepatotoxic effects were demonstrated [103]. Subsequently, the tetrahydronaphthopyrone derivative (15; FPL 52845), an early chemical lead, was structurally modified, and activity in the primary screens was optimized in the 5-hydroxy-10-propyl derivative (16; FPL 57787; proxicromil) [102]. *Table 1.1* shows how two major enhancements of activity in the dog hypotension screen may be achieved by (1) introduction of a cyclohexane ring fusion and (2) increasing lipophilicity by substitution with 5-hydroxy and 10-alkyl functionalities. The plasma levels of the two tetrahydronaphthopyrones (15 and 16) after oral dosing to dogs, as well as the plasma half-lives, are also recorded in the table. The data show that the poor propensity of chromone-2-carboxylate to be absorbed from the gastrointestinal tract can ultimately be overcome with highly lipophilic analogues such as proxicromil (16). It has been postulated that absorption may take place via ion-pair formation [104].

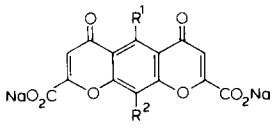
Proxicromil was taken forward for clinical evaluation and shown to be effective by the oral route against antigen [105] and exercise [106] challenge in patients. Therapeutic efficacy was also shown [107]. The compound was withdrawn, however, following the outcome of long-term toxicological investigations.

Over the last 5 years in our laboratories, there has been a resurgence of interest in polar dibasic acids for the topical (inhalation) treatment of asthma. The advantages offered by such compounds include the reduced chances of

Table 1.1. COMPARISON OF THE ANTIALLERGIC ACTIVITY AND ABSORPTION PROFILE OF THREE CHROMONE-2-CARBOXYLIC ACID DERIVATIVES

Compound	*FPL number*	*Dog hypotension*		*Rat PCA*		*log* $D_{7.4}$	*Plasma levels* [a] *(dog)*			$t_{1/2}$ *(h)*
		μg/kg per min for 15 min	*% inhibition*	*i.v.* ID_{50} *(mg/kg)*	*i.duo.* ID_{50} *(mg/kg)*		*1 h*	*3 h*	*5 h*	
Chromone-2-carboxylate	52839	200	15	> 10	(Inactive at 10 mg/kg)	− 1.5	n.d.[b]	n.d.	n.d.	n.d.
(15)	52845	50	58	0.4	10.7	+ 0.1	0.2	0.5	1.5	0.3
(16)	57787 (proxicromil)	20	73	6.4	15.5	+ 1.6	2	11	17	1.9

[a] Blood levels of compound, 1, 3 and 5 h after oral dosing at 20 mg/kg.
[b] n.d. = not determined.



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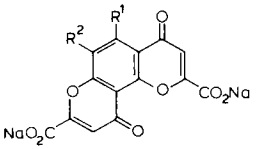

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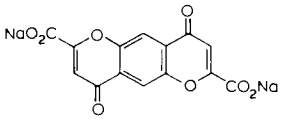
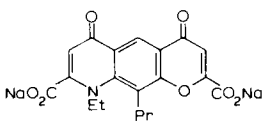


(19)

side-effects and toxicity resulting from the low dose which is normally adequate for topical treatment and the rapid renal and biliary excretion [108] which is ensured by the physicochemical properties of the molecules. A series of benzodipyran derivatives (17–19) was studied [109] and activity was optimized in the linear structure (17, R^1 = H, R^2 = Pr). The calcium salt of this compound (FPL 58668, probicromil) was later taken to the clinic and found to be effective in exercise [110] and antigen [111] challenge. Against antigen it showed a greater potency, efficacy and duration of action than did sodium cromoglycate in an asthmatic volunteer [46]. Therapeutic efficacy in seasonal rhinitis was also demonstrated [112]. Further clinical studies were halted because of a side-effect associated with the compound, namely a sensation of warmth (especially in the perineal region) experienced by some volunteers after inhalation. This effect has been shown by sodium cromoglycate, but only on i.v. infusion [108], and it is also a significant side-effect of the bisoxamic acid derivative, lodoxamide (see p. 42).



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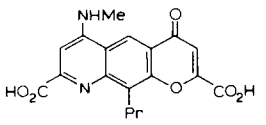
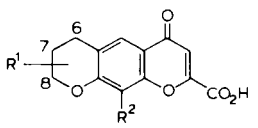


(21)

More recently, we have investigated pyranoquinolines [113, 114], two of which (20; FPL 59002; nedocromil sodium) and (21; FPL 59360; minocromil) have been taken further. Their activity in primary biological screens has been reported [115]; also, they appear to be devoid of side-effects and were shown

to inhibit antigen-induced bronchoconstriction in atopic as well as in asthmatic volunteers [115]. These compounds are at present undergoing clinical therapeutic trials.

Many other companies have studied chromone as a pharmacophore for antiallergic activity. Eisai claimed tricyclic pyranochromone-2-carboxylic acid derivatives (22) [116] closely related to proxicromil to be active, and appear to be taking the compound EAA (22, R^1 = 6,8,8-trimethyl, R^2 = Me) [117] through a development programme. Interestingly, the (+)-enantiomer was 2–3-times as potent as its antipode in rat PCA [117]. The synthesis of deuterated material (22, R^1 = 6,8,8-trimethyl, R^2 = CD_3) for metabolic studies has also been described [118].



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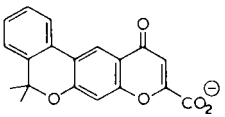


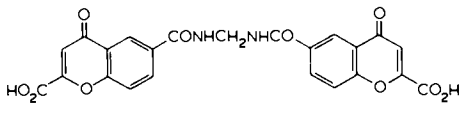
(23)

A series of benzopyranochromone derivatives [119] structurally derived from cannabinol was studied by Pharma-Research Canada. One of their more active compounds in rat PCA (23; PRD-92 Ea) was nominated for further investigation. Cross-tachyphylaxis with sodium cromoglycate in rat PCA and efficacy in a monkey asthma model were described [120]. Though the human pharmacokinetics of this compound were studied [121], no clinical efficacy data have been published. The compound has been shown to produce direct local irritant effects when injected intradermally or administered by aerosol in animals [122].

Other chromone-2-carboxylic acid derivatives which have been reported include bis-systems related to sodium cromoglycate but possessing amide linking chains, of which compound (24) was the most active in rat PCA [123].

Workers at Miles Laboratories demonstrated antiallergic activity for 5-(3-*p*-cyanophenoxy-2-hydroxypropoxy)chromone-2-carboxylic acid (25; TR 2815) and the corresponding tetrazole (26; TR 2855; cromitrile sodium). The latter compound was also shown to possess bronchodilator and anti-inflammatory activity [124] and its pharmacokinetics in animals have been studied [125].

Tetrazole is well known by medicinal chemists to mimic the carboxylic acid function [126] and several examples are known of tetrazolylchromones possessing activity comparable with the corresponding chromonecarboxylic acids.



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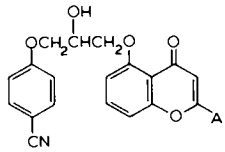
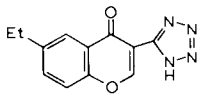


(25) A = CO_2H

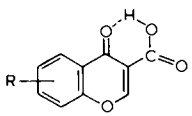


(27)

(26) A =

pK_a values for chromone-2-carboxylic acid and 2-(5-tetrazolyl)chromone are similar (1.62 and 1.68, respectively) [46]. Thus, simple 2-(5-tetrazolyl)chromones [127] as well as tetrazole analogues of sodium cromoglycate [128] and 6-ethyl-3-(5-tetrazolyl)chromone (27; AA-344; Takeda) [129] have all been shown to be inhibitors of rat PCA. The pharmacology [130, 131, 132] and metabolic fate [133] of the last compound have been reported, as well as the medicinal chemistry of analogues [134, 135]. In addition, brief mention has been made of its clinical effectiveness [129]. Other tetrazoles which possess antiallergic activity have been described in a review [126].

Chromone-3-carboxylates (28) (in contrast to both 3-tetrazolylchromones and chromone-2-carboxylic acids) are devoid of antiallergic activity as measured by the rat PCA test [134]. This has been attributed to the weak acidity [136] of the molecule resulting from hydrogen bonding of the acid function to the 4-oxo group [134] as shown. A pK_a value of 8.9 has been claimed for chromone-3-carboxylic acids, but we have determined the parent compound (28, R = H) to have a more likely value of 4.0 using a conductometric method [46]. The absence of activity in this series may alternatively be explained in terms of its lack of bioavailability at the site of action, for example, if ready *in vivo* decarboxylation were taking place. This explanation seems more



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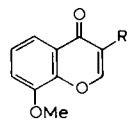
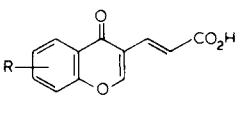


(29) R = CH_2OH

(30) R = CO_2H

feasible, since 3-hydroxymethyl-8-methoxychromone (29; W-8011; Warner-Lambert), which shows antiallergic activity *in vivo*, appears to act as a prodrug of the corresponding 3-carboxy derivative (30) [137]. Thus, the hydroxymethyl compound (29) was orally effective in rat PCA and inhibited active cutaneous anaphylaxis in the same species as well as partially blocking dyspnoea and coughing in sensitized guinea-pigs exposed to aerosolized antigen [138]. However, the compound did not inhibit release of histamine from rat mast cells *in vitro*, whereas its known metabolite (30) was an effective inhibitor of anaphylaxis *in vitro* [137].

Chromone-3-acrylic acids also possess antiallergic properties. A series of *trans* (but not *cis*) compounds (31) was active in rat PCA, but only the 6-isopropyl derivative was more potent than sodium cromoglycate [139]. Other acid functions which have been shown to be acceptable alternatives to carboxylate for antiallergy activity when attached to a chromone nucleus include



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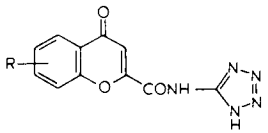

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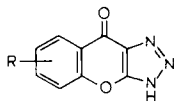

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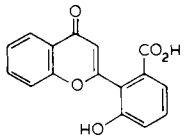

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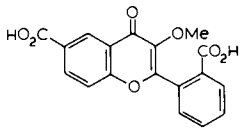
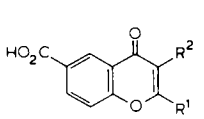
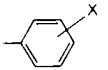


(35)

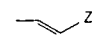
N-tetrazolylcarboxamide (a series of analogues (32) was studied by Allen and Hanbury's) [140] and a fused triazole ring (Beecham's have in recent years been investigating compounds of type (33)) [141]. Flavone derivatives (such as (34) and (35)) substituted on the phenyl ring with an acidic function possess weak rat PCA activity [142, 143].

Chromones in which a carboxylic acid group resides in the 6-position (compare with the structure of active xanthones, described later) were also shown to possess antiallergic activity. Thus, Carlo Erba Research have demonstrated that 2-aryl (36) [144], 2-heteroaryl [144], *trans*-2-cyclopropyl (37) [145] and *trans*-2-ethenyl (38) [146] derivatives of chromone-6-carboxylates are moderate inhibitors of rat PCA by the oral route and are effective *in vitro* against histamine



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

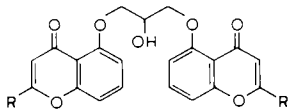
(37) R^1 = 

(38) R^1 = 

release from rat peritoneal mast cells. Activity was optimal for flavones (36) when X = 2-alkoxy. One derivative (36, X = 2-OiPr; K 10149; isocromil) was evaluated by the oral route in patients with nasal atopy [147]. A single dose of 600 mg administered to 20 subjects significantly inhibited nasal conductance changes to antigen. In a therapeutic study in 40 patients, the drug (300 mg given three times daily for 1 week) effectively protected patients from hay fever symptoms. Nevertheless, the development of this compound appears to have been discontinued.

Substitution of the chromone nucleus in the 2-cyclopropyl and 2-vinyl series by a 3-propyl or 3-propoxy substituent increased potency, and this was greatest for compound (37) when Y = 2-methylphenyl, R^2 = *n*-propyl and compound (38) when Z = 5-methyl-2-furyl, R^2 = *n*-propyl.

The ability of acid substituents attached to the 2-, 3- or 6-position of the chromone nucleus to produce antiallergic compounds is inconsistent with a stereospecific role for this group at the sodium cromoglycate receptor. In



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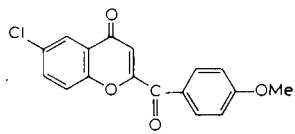
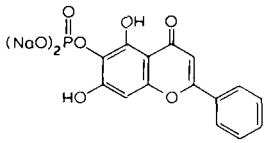


(40)

addition, activity in rat PCA was shown for a series of neutral analogues (39, R = methyl, aryl, heteroaryl) of sodium cromoglycate and corresponding salicylic acid derivatives [148]. A series of 2-aroylchromones were active in rat PCA, and compound (40) had a potency similar to that of sodium cromoglycate but was also orally active [149].

Several publications have described the antiallergic properties of naturally occurring flavone derivatives (the bioflavonoids) or their simple derivatives. Notably, baicalein disodium 6-phosphate (41; Takeda) [150] was shown to be effective in several *in vitro* and *in vivo* models of hypersensitivity in the rat, guinea-pig and monkey [151]. Several natural flavonoids were found to inhibit secretion from rat mast cells and rabbit neutrophils [152].



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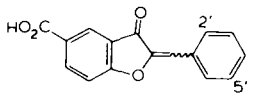
BENZOFURANONES

Carlo Erba researchers have studied a series of 2-benzylidene-3(2*H*)-benzofuran-5-carboxylic acid derivatives (42) which have structural features similar to these of the flavone-6-carboxylic acids (36) already described. Many of the compounds tested were more potent than sodium cromoglycate i.v. in

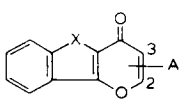


(42)

the rat PCA and were also orally active [153]. The most potent compounds had alkoxy substituents in the 2′- and 5′-positions. The stereochemistry about the double bond was unspecified.

COMPOUNDS CONTAINING THE PYRONE RING SYSTEM

A number of compounds structurally related to chromones which contain the 4-pyrone moiety fused to another heterocyclic system have been studied for antiallergic activity. Notably, several groups have investigated compounds which conform to structure (43), where A is an acidic substituent in the 2- or 3-position of the pyrone ring. It was reported from Warner-Lambert [154] that certain 4-oxopyrano[3,2-*b*]indoles (44, R^3 = alkyl or phenyl, A = carboxylic acid, tetrazole or *N*-tetrazolylcarboxamide) possess activity on intraperitoneal injection in rat PCA of similar potency to sodium cromoglycate, but they were also orally active. The tetrazoles were generally the most active compounds, but otherwise no clear structure-activity relationships emerged on altering the substituents R^1, R^2 and R^3. The Upjohn Company reported the ability of benzothieno- (43, X = S, A = 2-CO_2H) and benzofuranopyrans (43, X = O, A = 2-CO_2H) to inhibit rat PCA as well as Prausnitz-Kustner (PK) reactions



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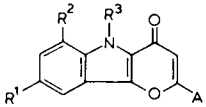
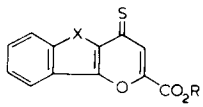


(44)

and active cutaneous anaphylaxis (ACA) in rhesus monkeys [155]. The more active sulphur compound was 3.2-times as active as sodium cromoglycate against the PK reaction and 18-times as active against ACA.

Closely related compounds have been described, including pyranoindoles (43, X = NMe or NEt, A = 2-CO_2H) [156], tetrazole derivatives (43, X = CH_2, O, S, NMe or NEt, A = tetrazol-5-yl) [157, 158] and benzofurano- and benzothienopyran derivatives (43, X = O or S, A = 2-CO_2H, 3-$COCO_2H$ or 3-CO_2H) as well as the 4-thioxo analogues (45, X = O or S) [159]. Several



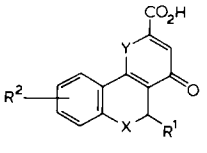
(45)

of these compounds, as well as the indane derivative (43, X = CH_2, A = 2-CO_2H), have been tested (i.v.) in rat PCA [160] and the results are shown in *Table 1.2* where it can be seen that activity is generally similar to that of sodium cromoglycate.

A series of 4*H*,5*H*-pyrano[3,2-*c*][1]benzopyran-4-one derivatives (46, X = Y = O) as well as the corresponding benzothiopyrano analogues (46, X = S or SO_2, Y = O) and pyridones (46, X = S or SO_2, Y = NH) from Ayerst Laboratories has been described [161]. Activity (i.v.) in rat PCA was poor for all compounds, although the pyridones (46, Y = NH) were somewhat

Table 1.2. RAT PCA ACTIVITY OF FUSED PYRANS

Compound	*Rat PCA; i.v. ED_{50} value (mg/kg)*
Sodium cromoglycate	2.5
(43) X = CH_2 A = 2-CO_2H	1.6
(43) X = NMe A = 2-CO_2H	1.5
(43) X = O A = 2-CO_2H	4.0
(43) X = S A = 2-CO_2H	5.0



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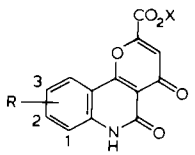
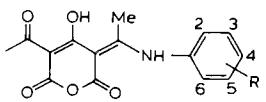


(47)

more active than the pyrone derivatives. The potassium salt of the pyrone derivative (46, $R^1 = R^2 = H$, X = Y = O) was the only structure to possess moderate oral activity (ED_{50} value = 10 mg/kg) and surprisingly (contrast with chromones, see p. 18) the tetrazole derivatives of (46) were essentially inactive. In a closely related series, greater potency was demonstrated by workers at Mitsubishi Yuka Pharmaceuticals for pyranoquinolinecarboxylic acids (47, X = H) and their corresponding aliphatic C_1-C_6 esters (47, X = alkyl) [162]. Some of the esters had significant oral activity. One derivative (47, R = 3-Me, X = H; MY-5231) has been investigated in several models of allergy; in particular, it was more potent than sodium cromoglycate *in vitro* against release of histamine from passively sensitized monkey lung [163].

Pyranenamines (48) have been extensively investigated by workers at Smith, Kline & French Laboratories. A series of approximately 100 derivatives was tested in rat PCA and also *in vitro* in rat and primate fragmented lung assays [164]. Attempts to optimize activity using a Topliss approach were unsuccessful, but using a three-dimensional graphical technique (in which activity was plotted against σ and π values for substituents R) a strong dependence of activity on hydrophilicity was established [165]. This led to the synthesis of



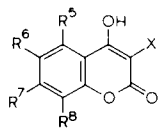
(48)

compounds which were an order of magnitude more potent, notably the compound (48, R = 3-NH_2-4-OH; SKF-78729A) which had i.v. activity similar to that of sodium cromoglycate in the PCA test and was also orally active. It has been studied in some detail [166, 167] and was reported to be under investigation in clinical trials against allergic rhinitis and for the prevention of acute attacks of asthma [168]. Application of QSAR methods (regressional analysis) led to further enhancement of activity, culminating in a

compound (48, R = 3,5-di-NHCOCH(OH)CH_2OH) which was 1000-times as active as SKF-78729A by the i.v. route in rat PCA [165].

COUMARINS AND ISOCOUMARINS

3-Nitro- [169] and 3-cyano-4-hydroxycoumarins [170] (49, X = NO_2, CN) with antiallergic activity have been described by Beechams. In both series, activity was optimized in the rat PCA test (by the subcutaneous route) when the substituents R^6 and R^7 were alkyl groups (methyl or ethyl). This corresponds to the findings by the same group for indanedione and dioxonaphthotriazole derivatives (see pp. 31 and 47, respectively). Nitrocoumarins (49, X = NO_2) were subjected to regressional analysis (using Hansch equations) and CNDO/2 molecular orbital calculations [171]. This led to the synthesis of the most active compound in the series, the 7-isopropyl derivative (49, X = NO_2, R^7 = isopropyl).



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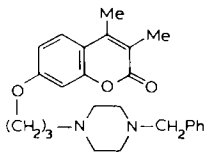

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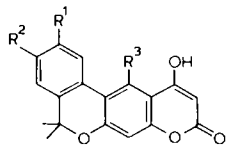

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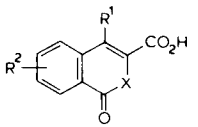
The piperazinopropoxycoumarin (50; BM 15, 100; Boehringer-Mannheim), a basic compound, has antihistaminic properties, as would be expected from its structure, but was claimed to exert its antiallergic activity chiefly through inhibition of mediator release from tissue mast cells. The *in vitro* [172, 173] and *in vivo* [174] antiallergic profile is not inconsistent with this claim. By comparing BM 15,100 with mepyramine in the PCA test, it was concluded that its efficacy in this model was too great to be explained simply on the basis of H_1-antagonism [174]. In addition, a single oral dose (20 mg) of the compound significantly protected asymptomatic volunteers with allergic asthma against bronchial antigen challenge [175]. The development of this compound was discontinued following safety evaluation studies in animals. 4-Hydroxycoumarins (51) related to the chromone PRD-92Ea (23) have also been reported from Pharma-Research Canada to possess antiallergic activity [176]. In addition to their ability to inhibit rat PCA, the compounds antagonized contractions induced by mediators (histamine, serotonin, bradykinin, PGE_2, $PGF_{2\alpha}$ and SRS-A) *in vitro* in smooth muscle preparations (e.g., guinea-pig ileum strips). In the PCA

test, the compounds were generally less potent than the corresponding chromones. Activity was maximal when a hydroxyethoxy group was present (51, $R^2 = R^3 = H$, $R^1 = O(CH_2)_2OH$).

The isocoumarin-3-carboxylic acids (52, X = O) as well as the corresponding thiaisocoumarins (52, X = S) and isoquinolinones (52, X = NH) have been claimed by Beecham's in the patent literature [177] (PCA data were given, but compounds were of low potency), though no development compound appears to have emerged from this chemical area. The isocoumarin derivative (53; F-1375; Pierre Fabre) reduced compound 48/80-induced histamine release from rat peritoneal mastocytes [178]. Similar activity has been demonstrated



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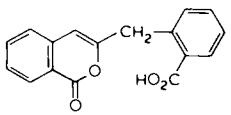

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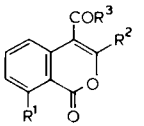

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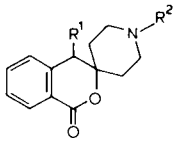

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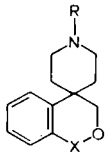


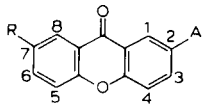
(56)

for a series of 4-acylisocoumarins (54) related to oosponol [179]. One of these derivatives (54, $R^1 = R^2 = H$, $R^3 = 4\text{-}C_6H_4\text{-}CO_2H$; KIT-302; Ikeda) has been investigated further [180]. Spiro [isochroman piperidine] analogues (55, R^1 = H or CO_2Et and 56, X = CO or CH_2) have also been studied against release of histamine induced by compound 48/80 from rat peritoneal mast cells [181]. Structure-activity relationships were discussed, but compounds lacked potency in this test. A number of related spiro-heterocycles have also been synthesized and shown to have some activity [182].

XANTHONES

Of all the heterocyclic systems which have been studied for antiallergic activity, only chromone has received more attention than xanthone as a pharmacophore. It seems that with few exceptions xanthones require an acid function in the 2-position to express activity. Many compounds conform to structure (57), where A is a carboxylic acid or tetrazole group.

In a series of 7-alkoxy substituted xanthones synthesized in the laboratories of Allen and Hanbury's, three compounds (57, R = OMe, A = CO_2H; AH-6556), (57, R = OMe, A = CN_4H; AH-7079), (57, R = $O(CH_2)_2OH$, A = CO_2H; AH-7725) were of special interest. They were studied in several *in vitro* models of allergy against histamine release from sensitized preparations of human and guinea-pig chopped lung and rat peritoneal mast cells [183, 184]. In these models they behaved similarly to sodium cromoglycate, but were also

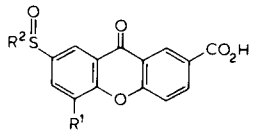


(57)

capable of inhibiting histamine release from the leucocytes of asthmatic patients. They were active, too, *in vivo* in rat PCA and passive peritoneal anaphylaxis [184]. On inhalation, AH-7079 was shown to inhibit asthmatic attacks induced by bronchial antigen challenge in 7 out of 12 patients [183]. AH-7725 was effective by mouth, given as a single dose of 500 mg 2 h before antigen challenge, as an inhibitor of the immediate but not the late asthmatic reaction [185].

The Schering-Plough Corporation, using AH-7725 as a lead, synthesized a number of compounds in which the 7-alkoxy chain was extended (57, A = CO_2H, R = $OCH_2CH(OH)CH_2XR$, where X = O, S or SO) [186]. Using the rat PCA test as a basis, the compounds were submitted to a regressional analysis which showed a dependence of activity on the bulk of the substituent R. Both molar refractivity (MR) and the Taft parameter (Es) were highly significant in the regression equation. Only the compound (57) (R = $OCH_2CH(OH)CH_2SMe$) was orally active.

Syntex researchers studied a series of xanthone-2-carboxylic acids containing a single methoxy substituent [187]. Activity (i.v.) in the rat PCA test fell in the order 5-OMe $\approx$ 7-OMe > H > 8-OMe > 6-OMe $\approx$ 3-OMe > 4-OMe > 1-OMe. Other 5- and 7-substituents (including ethoxy, hydroxy, fluoro, ethyl and carboxy) were shown to enhance activity. Another publication [188] describes the moderate PCA activity shown by dimethoxy-substituted and 7-acylxanthones as well as the greatly enhanced activity of derivatives (58), containing a sulphoxide group in the 7-position. Some of these compounds also inhibited rat intestinal anaphylaxis, suggested as a model of human food allergy [189]. This activity broadly correlated with activity in PCA. Two compounds have been studied further: the sulphoxide, tixanox (58, R^1 = H, R^2 = CH_3;

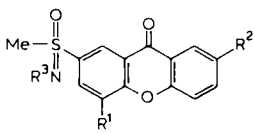


(58)

RS-7337) and xanoxic acid (57, R = OiPr, A = CO_2H; RS-7540) were both found to be orally effective in exercise challenge [190–192], and the former compound protected against human bronchial antigen challenge by the inhalation route [193]. Related dibenzotropone- and dibenzosuberonecarboxylic acids were shown to be bronchodilators in the guinea-pig [194]; however, no data on the antiallergic properties of these compounds were given.

Xanthone-2,7-dicarboxylic acid (57, A = R = CO_2H) was active by the i.v. [188] and i.p. [195] routes, but not orally. A group at Merrell-National Laboratories showed that the corresponding biscarboxamides (but not the bisethyl ester) did, however, show significant oral activity in rat PCA, the most active compound being the bisdimethylamide [195]. Tertiary amides were more active than secondary amides, but otherwise no relationship between the physicochemical properties of the groups and potency in rat PCA was evident.

Roussel scientists studied sulphoximide derivatives (59) for their ability to inhibit rat PCA [196]. The initial hypothesis that this group could act as an isostere for the carboxylic acid function was not borne out, since compound (59, $R^1 = R^2 = R^3 = H$) was inactive. Nevertheless, xanthone-2-carboxylic acid derivatives possessing a 7-sulphoximido group (59, $R^2 = CO_2H$) were

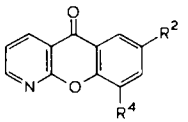


(59)

potent compounds and some were orally active. In particular, the structure (59, R^1 = n-C_6H_{13}, $R^2 = CO_2H$, $R^3 = H$; RU-31,156) was 260-times as potent as sodium cromoglycate by the i.v. route and has been studied in some detail [197]. Administered as the tromethamine salt, RU-31,156 (xanoxadate tromethamol or sudexanox tromethamine) was orally active in the rat PCA test (ED_{50} value = 0.19 mg/kg), and partially inhibited an IgG-mediated PCA reaction and anaphylactic bronchoconstriction in the rat. It also showed cross-

tachyphylaxis with sodium cromoglycate in the rat (IgE) PCA test, indicating that it shares a similar mode of action [198]. At doses of 0.4–1.5 mg/kg in children and 0.2–1.0 mg/kg in adults, RU-31,156 was effective in about 66% of extrinsic asthma sufferers [199].

Two active azaxanthone derivatives (60) were described by Yoshitomi. The tetrazole derivative (60, R^2 = 5-tetrazolyl, R^4 = Cl; Y-12,141 traxanox sodium) [200] was 5-times as potent as cromoglycate i.v. in rat PCA and was also orally active in this test [201]. The compound was active in experimental asthma in the guinea-pig (IgG mediated) [201] and also *in vitro* against antigen-induced histamine release from guinea-pig sensitized lung [201] and passively sensitized



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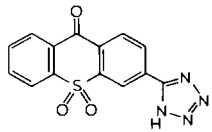
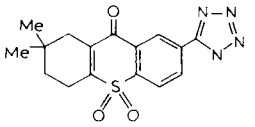


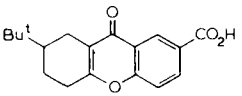
(61)

rat mast cells [202]. The non-acidic derivative (60, R^2 = COMe, R^4 = H; Y-9,000) inhibited both IgE-mediated reactions in the rat (after i.p. and oral administration) and IgG-mediated systemic anaphylaxis in the mouse [203]. The possibility exists, though this has not been claimed by the authors, that Y-9,000 acts as a prodrug for the corresponding acid (60, R^2 = CO_2H, R^4 = H). The thioxanthone dioxide, doxantrazole (61; Wellcome) [204], is unusual in that it has a xanthone structure with an acidic function (tetrazole) in the 3-position of the heterocyclic system; yet intravenously in rat PCA, the compound was as active as sodium cromoglycate, and it was also orally effective [205]. Inhibition of anaphylactic bronchoconstriction in the rat was also reported. *In vitro*, doxantrazole inhibited histamine release from rat peritoneal mast cells taken from actively sensitized animals and from passively sensitized human chopped lung. It also inhibited cAMP phosphodiesterase, suggesting that its antiallergic effects may in part be related to an increase in intracellular cAMP concentrations [205]. Doxantrazole has given variable results in the clinic. An oral dose of 200 mg partially inhibited the immediate, but not the late, reaction when the drug was taken 1 h before bronchial antigen provocation [206], while in a further trial only 4 out of 15 patients were protected [207]. Exercise-induced asthma was not inhibited 1 h after 200 or 400 mg doxantrazole [208], but the compound appeared to be effective in some cases of cold urticaria [209]. In a trial involving 37 extrinsic asthmatics, subjective and objective improvements were reported after oral treatment with

doxantrazole (200 mg three times daily for 1 week) [210], but in a subsequent longer-term study in 14 patients, no effect was demonstrated by the drug given at the same dose for 1 month [211]. Doxantrazole was not developed beyond this stage, and an early indication of its poor therapeutic potential may have been its inability to inhibit the late reaction following bronchial antigen challenge. The carboxylic acid derivative (BW437C) related to doxantrazole was more potent as an inhibitor of antigen-induced histamine release from human leukocytes of 16 pollen-sensitive patients [212].

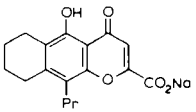
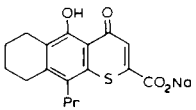
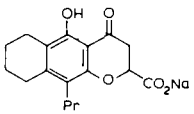
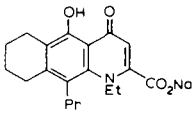


(62)



(63)

Table 1.3. COMPARATIVE ACTIVITY OF PROXICROMIL WITH THREE HETEROCYCLIC ANALOGUES

FPL	*Structure*	*Rat PCA*	*Dog hypotension*
		% inhibition at 10 mg/kg i.v.	*% inhibition*[a]
57787		36	73
58877		33	39
58770		10	26
58925		0	0

[a] Of sodium cromoglycate responses (AUC over 3 h) after infusion of 20 μg/kg per min for 15 min.

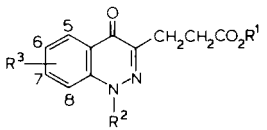
Two tetrahydro compounds of the xanthone type (62; TR-6078; Miles [124] and 63; K-4365; Kowa [213]) have also been claimed to have antiallergic properties. TR-6078 was also reported to possess bronchodilator activity [124].

THIACHROMONES, CHROMANONES AND *N*-ALKYLQUINOLINONES

In our own laboratories, we have compared FPL 57787 (proxicromil) with the three closely related heterocyclic systems shown in *Table 1.3* [46]. In both rat PCA and dog hypotension screens, activity falls in the order chromone > thiachromone > chromanone ≫ *N*-ethylquinolinone, the latter system being devoid of activity. There appears to be no simple explanation for these differences in activity.

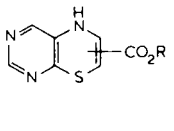
CINNOLINES

Despite several claims in the patent literature that cinnolines possess antiallergic activity, only one paper describing the medicinal chemistry of derivatives has appeared [214]. A series of cinnolone-3-propionic acids and their corresponding esters (64) was synthesized at ICI and shown to inhibit rat PCA.

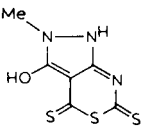


(64)

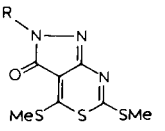
Simple, uncrowded alkyl substituents in the 6-position optimized activity, the most potent compounds being (64, $R^1 = R^2 = H$, R^3 = 6-Et) and the corresponding ethyl ester. This accords with the corresponding 3-tetrazolylchromones (see p. 18). Substitution of positions 5, 7 or 8 gave no improvement in activity, and bulky or strongly electron donating groups (alkoxy) in the 6-position were deleterious. The esters (64, R^1 = Me, Et, hexyl, etc.) were generally similar to the corresponding acids when administered intravenously, suggesting that they were rapidly hydrolysed *in vivo*. Oral activity was seen with several compounds, notably the ethyl ester (64, R^1 = Et, R^2 = Me, R^3 = 6-Et).



(65)



(66)



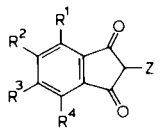
(67) R = H, Me, Ph

THIAZINES

Fused thiazine derivatives (65 [215], 66 [216], and 67 [216]) have been claimed to possess activity in rat PCA.

INDANEDIONES

Workers at Beecham Research Laboratories have described activity in rat PCA of 2-nitroindane-1,3-diones (68, $Z = NO_2$) [217]. The unsubstituted compound (68, $Z = NO_2$, $R^1 = R^2 = R^3 = R^4 = H$) had activity similar to that of sodium cromoglycate. Substitution on the benzene ring generally had little effect on activity, or in some cases reduced it. The 4,5-fused systems (68, $Z = NO_2$, $R^1R^2 = (CH=CH)_2$ or $(CH_2)_4$, $R^3 = R^4 = H$) and the tetrachloro



(68)

analogue (68, $Z = NO_2$, $R^1 = R^2 = R^3 = R^4 = Cl$) were inactive. Only the 5,6-disubstituted derivatives gave increased activity over the parent system. 5,6-Dimethyl-2-nitroindane-1,3-dione (68, $Z = NO_2$, $R^1 = R^4 = H$, $R^2 = R^3 = Me$; BRL 10833; nivimedone) was the most potent compound synthesized, being 40-times as active as sodium cromoglycate in rat PCA. Nivimedone inhibited antigen-induced release of histamine and SRS-A from passively sensitized human chopped lung at concentrations similar to those required for inhibition by sodium cromoglycate, but was more potent as an inhibitor of histamine release by antigen from actively or passively sensitized rat peritoneal cells and from passively sensitized rat skin [218]. Nivimedone was shown to produce a significant protective effect against the immediate reaction following bronchial antigen challenge in (a) 5 out of 10 asthmatics following 40 mg by inhalation [219] and (b) 5 out of 9 patients after 2 mg/kg orally and 2 out of

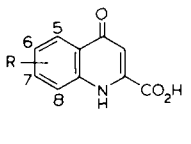
10 patients after 20 mg/kg orally [220]. Therapeutic efficacy of nivimedone in bronchial asthma was demonstrated in a 6-week double-blind crossover trial in 24 patients given an oral dose of 200 mg, thrice daily [221]. This is perhaps the only published clear demonstration of therapeutic efficacy for an antiallergic drug other than sodium cromoglycate. Unfortunately, chronic toxicity studies in the rat revealed a dose-related incidence of malignant bladder tumours, which resulted in further studies on this compound being halted [221].

2-Cyanoindanediones (68, Z = CN) have also been studied at Beecham's [170]. Structural requirements for activity in rat PCA with these compounds were similar to those for the nitroindanediones.

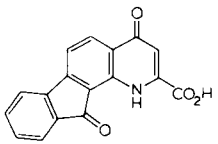
QUINOLINE DERIVATIVES

Given the structural similarity between chromones and their nitrogen analogues, it is not surprising that carboxylic acids of quinoline and quinolinone have been widely investigated as antiallergic agents.

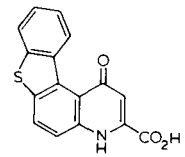
Simple 1,4-dihydro-4-oxoquinolinecarboxylic acids (69) have been reported from Riker Laboratories [222]. Significant activity in rat PCA was achieved only when electron-withdrawing groups (MeCO, PhCO and $PhSO_2$) were present in the 7- or 8-positions. The indenoquinoline (70) was as potent as



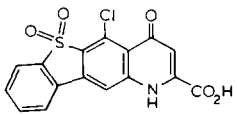
(69)



(70)



(71)



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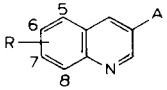
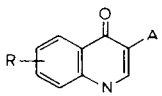


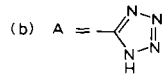
(73)



(74)

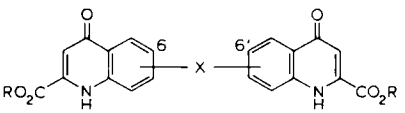
(a) A = CO_2H

(b) A =



sodium cromoglycate and inspired the synthesis of the six possible isomeric benzothienoquinolines [223] and the corresponding sulphoxide and sulphone derivatives, of which the 5,6-fused sulphoxide (71) and the 5-chloro-6,7-fused sulphone (72) were the most active compounds, being approximately 8-times as potent as sodium cromoglycate given i.p. These compounds were not orally active. Quinolines (73) and quinolinones (74) substituted in the 3-position by an acid function (tetrazole or carboxylic acid) were also shown by the same company to possess activity in rat PCA [224]. Generally, tetrazoles were more active than carboxylates and the desirability of having a 4-oxo substituent was demonstrated by the greater potency of the quinolinones. Activity of tetrazolylquinolinones (74b) was optimized by introduction of a (– I + R) substituent in the 8-position, the isopropoxy and chloro derivatives being 33-times as potent as sodium cromoglycate (i.p.). These compounds were also orally active, but transfer of the substituents to the 6-position led to a dramatic loss in activity. Exactly the opposite effect was found with ethyl as a substituent; 8-Et was much less potent orally than 6-Et.

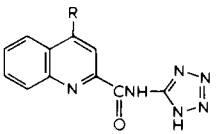
1,4-Dihydro-4-oxoquinoline-3-carboxylates (74a) as well as the isomeric 2-carboxylates (69) and their corresponding esters have been studied at Upjohn [225]. It was found that amino-substitution gave the most potent compounds; structure (69, R = 7-NH_2, 8-Me) was 25-times as active as sodium cromogly-



(75)

cate i.v. in rat PCA. Bis-linked quinolines (75) were also active [225]. A direct 6,6′ linkage conferred greatest activity on these dibasic acids. Though no biological data were given, simple quinolinones (69) and bis-linked compounds (75) have also been claimed by Liu, Lu and Lee as antiallergic agents [226].

Several *N*-tetrazolylquinoline-2-carboxamide derivatives (76, Allen and Hanbury's) were examined for antiallergic activity in rat passive peritoneal



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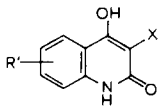
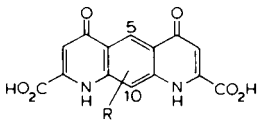


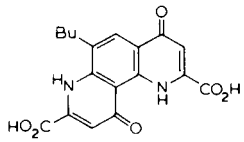
(77)

anaphylaxis, isolated rat peritoneal mast cells, and human lung fragments [227]. The most interesting compound of this series (76, R = OMe; AH-9679) was shown to inhibit antigen-induced histamine and in particular SRS-A release from canine lung [228]. A series of non-acidic derivatives related to 3-amino-4-hydroxyquinolin-2(1H)-one (77, X = NHR; Tanabe) and the corresponding oxazolo-fused systems showed weak PCA activity [229]. Related 4-hydroxy-3-nitro-2-quinolinones (77, X = NO_2; Beechams) corresponding to the coumarins (49) were active in rat PCA by the subcutaneous route [230] and simple quinoline derivatives with potential as antiallergic agents have been synthesized by Goerlitzer [231].

A number of pyridoquinolinedicarboxylic acids have been investigated in some detail (compare with benzodipyrans already discussed on p. 16) and represent the most interesting quinoline compounds which have been studied for antiallergic activity. Both Upjohn [232] and ICI[233] have described very closely related linear tricyclic systems (78) and their angular isomers. Although no simple structure-activity relationships were evident from results in rat PCA, the compounds described by Upjohn were generally much more potent than simple quinaldic acids (69). One of the compounds (78, R = 10-Cl; U-38,650),



(78)



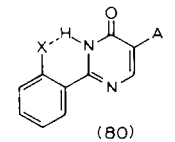
(79)

which was 50-times as potent as sodium cromoglycate, was studied in other models of allergy in rats and monkeys [234]. The most potent compound described by Upjohn, a butyl-substituted angular structure (79), was predated in the patent literature by ICI and developed by them under the approved name bufrolin (ICI 74,917) [235]. Pharmacological studies showed that this compound was a potent inhibitor of immediate hypersensitivity reactions in the rat, mouse and guinea-pig [236]. In the clinic, bufrolin delivered intranasally was shown to inhibit the effects of nasal antigen challenge [237, 238] and also demonstrated limited efficacy in seasonal allergic rhinitis [239]. In a study designed to investigate efficacy in asthma, 13 asthmatic patients were treated with a placebo for 4 weeks followed by bufrolin (1 mg by pressurized aerosol four times a day) for 4 weeks [240]. Antigen challenge tests were also performed during the trial, in every case 15 min after drug treatment. Though the drug significantly reduced the antigen-induced fall in peak expiratory flow rates, the

patients failed to show any clinical improvement in their asthma during the 4-week active treatment period. This study elegantly shows that a compound which is effective against bronchial antigen challenge is not necessarily of therapeutic value in asthma.

PYRIMIDINES AND QUINAZOLINES

A series of 2-arylpyrimidinones (80; Bristol Laboratories) has been extensively studied [241, 242]. The tetrazole derivatives (80b) were generally 5–10-times more potent than the corresponding carboxylic acids (80a) and high activity



(80)

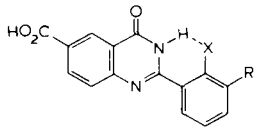
(a) A = $-CO_2H$

(b) A = 

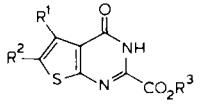
was achieved in rat PCA only when the ortho substituent on the phenyl ring was capable of hydrogen bonding to the NH of the pyrimidine ring (as shown in structure (80)), especially when X = alkoxy. Many of these structures had considerable oral activity. The compounds closely resemble the azaxanthines, which require a similar hydrogen bonding capability for optimization of activity. The pyrimidinonecarboxylic acids (80a) were subjected to Free-Wilson and Hansch analysis which showed that activity depended in a parabolic way on lipophilicity and confirmed the importance of the hydrogen bonding capability [243].

The most potent of the pyrimidinones (80b, X = OPr; BL-5255) [244] was studied further in models of immediate hypersensitivity. By the oral route it was a potent inhibitor of rat PCA, rat PPA, antigen-induced airway constriction in actively sensitized rats [245], and IgG mediated reactions in the rat and guinea-pig [246]. Antigen-induced release of histamine and kallikrein from passively sensitized human chopped lung was also inhibited by BL-5255 and the compound was 4-times more potent than theophylline in inhibiting rat lung cAMP phosphodiesterase [247]. Further experimental results have recently been reported [248] in which mediator release from passively sensitized mast cells in the rat peritoneal cavity, monkey chopped lung, or human chopped lung tissue was inhibited by BL-5255. The compound is undergoing therapeutic

evaluation in asthma; well-tolerated single doses of the drug generated high plasma levels (up to 40 μM) and potentially effective levels persisted for as long as 8 h [248]. 2-Aryl-3,4-dihydro-4-oxo-6-quinazolinecarboxylic acids (81; Carlo Erba) which were active orally in rat PCA, again required the hydrogen bonding capability, as previously described, for significant activity to be shown [249]. The most potent compound was the dialkoxy derivative (81, X = OEt,



(81)

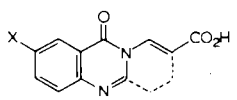


(82)

R = OMe), which was also 5-times as potent as sodium cromoglycate as an inhibitor of histamine release from passively sensitized rat peritoneal mast cells *in vitro*.

Thienopyrimidinones (82; Mead Johnson) also possessed oral antiallergic activity in rat PCA [250]. Potency of acids (R^3 = H) was optimized when R^1 = H or Me and R^2 = lower alkyl. Esters (R^3 = Et) were also orally active. related thien-2-yloxamates and thienooxazine-2-carboxylates were also investigated.

Oral activity in rat PCA has recently been described for 3-(4-oxo-4*H*-quinazolin-3-yl)-2-propenoic acids (83; Hoffman-La Roche) [251]. These compounds were considered structurally to be ring-opened derivatives of pyrido[2,1-*b*]quinazolinecarboxylic acids (see p. 43), as indicated by the dotted line in structure (83). Indeed, the *Z* isomer of one of the compounds (*cis*-(83), X = SMe) which does not fit this model (compare also with xanthones) was inactive in rat PCA. Also in common with the related ring systems, activity was optimized by the presence of an electron-donating function in the 6-position (83, X = alkoxy, alkylthio or alkyl). The most potent of this series of compounds was (83, X = SMe; Ro 22-3747), which has also been shown to be a potent inhibitor of anaphylactic bronchospasm in passively sensitized rats and antigen-induced histamine release from passively sensitized rat peritoneal



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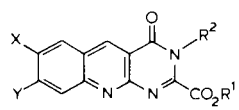


(84)

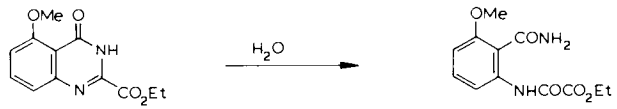
mast cells [252]. This compound, unlike sodium cromoglycate, also inhibited mediator release from antigen-challenged guinea-pig lung fragments [252]. The compound was scheduled for clinical evaluation as an oral antiallergic.

Structural modification of simple 3,4-dihydro-4-oxoquinazoline-2-carboxylates led to a new ring system, viz., 3,4-dihydro-4-oxopyrimido[4,5-*b*]quinoline-2-carboxylate (84; Pfizer) with oral antiallergy (rat PCA) activity [253]. A series of more than 50 derivatives was synthesized [253], showing that esters (R^1 = Et) were preferable to the corresponding acids, that introduction of substituents in the 3-position (e.g., R^2 = Me) was detrimental to activity, and that electron-donating substituents, particularly in the 6- and 7-positions (X and Y = alkoxy) gave the most active compounds. A U.S.-approved name, pirolate, was allocated to the 7,8-dimethoxy compound (84, R^1 = Et, R^2 = H, X = Y = OMe; CP-32,387) [254], but no clinical data have appeared.

Other quinazoline derivatives are considered in the section pertaining to nitrogen bridgehead compounds.

OXAMIC ACIDS

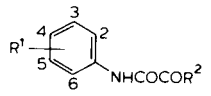
During an investigation at Wyeth Laboratories of the antiallergic activity of the quinazoline derivative (85), it was found that different batches of the compound showed variable intraperitoneal activity in rat PCA [255]. This was ultimately shown to be due to the presence of variable amounts of a contaminant, the ethyl oxanilate (86) derived from the parent compound by hydrolytic cleavage. This



(85) (86)

latter compound was subsequently synthesized and found to be considerably more potent than the quinazoline [255].

A large number of phenyloxamate derivatives (87) were synthesized and tested intraperitoneally (i.p.) and orally (p.o.) in rat PCA [255]. Replacement of the oxamate function by alternative groups (e.g., NHCOPr, $NHCOCH_2CO_2Et$, $CH_2NHCOCO_2Et$, $NHCSCO_2Et$, $CONHCOCO_2Et$) and other groups which were potential prodrugs of oxamic acid (e.g., NHCOCONHR, $NHCOCCl_3$) resulted in poor activity. Drastic loss of activity was also shown when the phenyl ring was replaced by various non-aromatic species (e.g.,H, cyclohexyl, piperidyl) or when the secondary amidic nitrogen

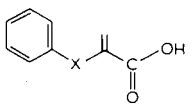


(87)

was replaced (e.g., by O or NMe) or omitted. Generally, then, only compounds (87, R^2 = Oalkyl, OH) and some heterocyclic analogues had interesting levels of activity. The various esters (R^2 = OEt, OPr, OBu, OcC_6H_{11}, etc.) of a given oxamic acid all had similar activity i.p. in rat PCA, implying that they are all acting as prodrugs of the acid with similar kinetics of hydrolysis. The acid corresponding to (86) was equipotent with the ester i.v. but much less active p.o., suggesting again that the ester is acting as a prodrug, and also that it is more readily absorbed than the acid from the gastrointestinal tract.

Substitution of the phenyl ring in (87) led to compounds with a wide range of activity, but no clear structure-activity relationships were evident. 2-Carbamoyl and 3-methoxy substituents were most beneficial to activity and compound (86; Wy-16,922; Wyeth) was the most interesting in this series and has been evaluated in several models of allergy [256]. Later papers from the same company have described potent oxamic esters and acids (87, R^2 = OEt, OH, R^1 = 2-CN-3-alkylamino) [257] and the corresponding tetrazoles [258]. From these structures, the sodium salt (87, R^2 = ONa, R^1 = 2-CN-3-NHMe; Wy-41,195) was chosen for further studies [259]. It was 520-times as potent as sodium cromoglycate in the rat PCA test (i.v.) and was also very effective by the oral route. Like sodium cromoglycate, it demonstrated tachyphylaxis in rat PCA and inhibited rat passive lung anaphylaxis and histamine-induced reflex bronchoconstriction in the dog [260]. Interestingly, the compound, also in common with sodium cromoglycate, reversed the hyperreactivity to methacholine induced by sulphur dioxide in the conscious dog [261].

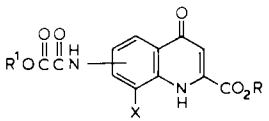
It has been pointed out [262] that many of the drugs which possess good antiallergic activity (e.g., chromones, quinolinones, quinazolinones, etc.) incorporate part-structure (88) within the framework of the molecule. In a theoretical study (using *ab initio* Hartree-Fock SCF calculations) of oxanilic acid and 2-carboxylic acids of quinolinone and chromone, it was shown that activity in rat PCA (assumed to be a direct measure of receptor binding of these



(88)

molecules) correlated well with the energy of the unoccupied antibonding π orbital which was the form of the lowest unoccupied molecular orbital (LUMO) in oxanilic acid (87, R^1 = H, R^2 = OH) [262]. The implication is that the drug acts as an electron acceptor in a charge-transfer complex with the receptor. This model is somewhat at variance with that proposed by Lunt [74] which stresses the importance of the 4-oxo substituent in chromones and quinolinones and its spatial relationship with the ring heteroatom and acidic function. The two models can, however, be partially reconciled when it is realized that potent antiallergic activity is observed for phenyloxamic acid derivatives only when the 2-position is substituted by an electronegative group which can supply a lone pair of electrons (87, R^1 = 2-Cl, $CONH_2$, CN, etc.) and may be fulfilling the role of the carbonyl function of the heterocyclic antiallergics.

Further theoretical studies by the Upjohn Company have recently extended molecular orbital calculations to bisoxamic acids [263] as well as pyridoquinolines (78), benzodipyran (17, R^1 = R^2 = H), quinoline-5-oxamic acids (89), pyridine-2,6-bisoxamic acid (90), benzotripyridine (91), and pyrimidine-4,6-bisoxamic acid (92) [264]. As in the earlier studies [262], the energy of the antibonding empty π orbital that was said to interact with a donor orbital on the receptor generally correlated with activity of the molecules in rat PCA



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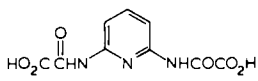

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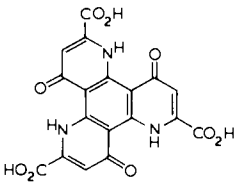

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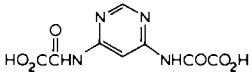

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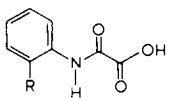
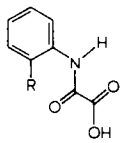


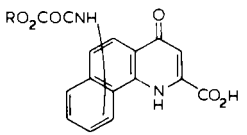
(93a) anti



(93b) syn

[264]. However, a number of structural factors had to be introduced to unify all the structural types. A detailed discussion of these papers is beyond the scope of this review, but one of the structural features considered was the conformation of the oxamic acid groups. Though evidence for some conformational flexibility was obtained from X-ray data, calculations showed that phenyloxamic acid is close to being a planar structure. This still allows an *ortho*-substituted oxamic acid to exist in two conformations (*anti*, 93a and *syn*, 93b). The *anti* conformation is favoured when R is not hydrogen (compounds studied with R = F, Cl or CN were all predicted to be locked in this form). 1,3-Bisoxamic acids could exist in *anti-anti, anti-syn* or *syn-syn* conformations. In order to obtain correlations between biological activity and the molecular orbital energies, it was important to take into account the contribution made by the various conformations for each molecule.

Some of the above considerations, coupled with the potent activity observed for pyridoquinolinedicarboxylic acids, led workers at Upjohn to synthesize (2-carboxy-1,4-dihydro-4-oxoquinolyl)oxamic acids (89) [265] and (2-carboxy-1,4-dihydro-4-oxobenzo[*h*]quinolyl)oxamic acids (94) [265] as well as the extremely potent *N*, *N'*-(phenylene)dioxamic acids (95) [266]. In particular, the diacid lodoxamide (95, R^1 = 2-Cl-5-CN, R^2 = H) had an ID_{50} value of 0.001 mg/kg in rat PCA (i.v.) and was therefore 2500-times as potent as sodium



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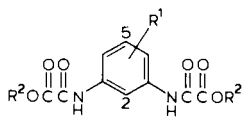


(95)

cromoglycate, probably the most potent compound reported in this test. The tromethamine salt of lodoxamide (U-42, 585) as well as the corresponding diethyl ester [267] (U-42, 718; lodoxamide ethyl) have both undergone extensive study [268].

Lodoxamide tromethamine was also orally active in rat PCA and the high potency of the compound was also evident from its ability to block histamine release from rat peritoneal mast cells challenged with compound 48/80 *in vitro* [269]. Intravenous doses as low as 0.001 mg/kg and oral doses of 5 mg/kg were capable of inhibiting respiratory frequency and tidal volume changes in naturally sensitive rhesus monkeys challenged with *Ascaris suum* antigen [270].

The biphasic or bell-shaped dose-response curve which has been demonstrated with sodium cromoglycate in various experimental studies (e.g., human

fragmented lung anaphylaxis [271] and rat peritoneal mast cells [272]) was also demonstrated with lodoxamide tromethamine using compound 48/80-induced histamine release from rat peritoneal mast cells [19].

Prior treatment with atropine produced some reversal of the loss of efficacy at high concentrations of lodoxamide, though this was not as clear cut as with sodium cromoglycate. However, this observation, together with the fact that high concentrations of lodoxamide were also shown to stimulate rat lung guanylate, in preference to adenylate cyclase, led the authors to conclude that stimulation of a cholinergic receptor was responsible for the decreasing activity of the drug above a certain optimal concentration [19].

The effectiveness of the tromethamine salt by the oral route is surprising in view of its high polarity and low pK_a, which would be expected to lead to minimal absorption from the gastrointestinal tract (compare with sodium cromoglycate). Even the diethyl ester, which may be better absorbed, would probably be hydrolysed to the bisacid and eliminated very rapidly from the blood. The extremely high potency of the compound may compensate for its probable poor bioavailability. Indeed, lodoxamide ethyl has been shown to be orally active in rat PCA (75% inhibition at 0.1 mg/kg) and in the *Ascaris*-sensitive primate (45% inhibition at 1 mg/kg) [273].

Considerable clinical investigation of the two compounds has been undertaken. It appears that the diethyl ester, which was intended for oral use, is no longer being developed due to unacceptable side-effects (see below) when the compound is administered by this route.

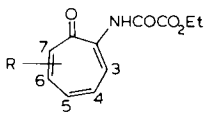
Like sodium cromoglycate, lodoxamide ethyl was ineffective as an inhibitor of compound 48/80-induced [274] or allergen-induced [275] wheal and flare reactions in the skin of human volunteers. Using oral doses of the same compound in 13 asthmatic patients, it was found that the provocation dose of antigen required to reduce the forced expiratory volume in 1 s by 20% (PD_{20}) increased 5-fold after a 1 mg dose, 6-fold after 3 mg, and 27-fold after 10 mg, when the drug was administered 30 min before bronchial antigen challenge [276]. More recently, similar results were obtained at 1 and 3 mg doses, but use of a 10 mg dose was stopped part-way through the trial because of a high incidence of side-effects. Doses of 0.3 mg were ineffective [277].

Lodoxamide tromethamine has been shown to inhibit exercise- [278, 279] and allergen-induced bronchoconstriction [280–284]. These latter studies all used the same technique of determining whether inhaled drug enabled the patients to tolerate significantly larger doses of antigen than placebo. Generally, the compound demonstrated a protective effect at a dose of 0.01 mg, but was more efficacious at 0.1 mg, 1 mg and 2 mg. In one study to determine its duration of action [283], lodoxamide tromethamine (0.1 mg) was administered

by inhalation to 15 subjects. Those subjects that were protected at 15 min (13 out of 15), were retested at 4 h, when 6 out of 13 were protected. Of the six patients, one was also protected at 8 h. Thus the duration of action of 0.1 mg lodoxamide by mouth is probably somewhat shorter than that of a therapeutic dose of sodium cromoglycate, though lodoxamide appears to be more potent, and increasing the dose (if this were tolerable) would almost certainly lengthen the duration. The drug appeared to have no significant effect on late reactions [282]. In one therapeutic study, lodoxamide tromethamine (0.1 mg, 0.5 mg) or placebo were given by inhalation four times a day for 4 weeks to 43 chronic asthmatics [285]. No therapeutic benefit could be demonstrated in this trial, though the authors comment that longer-term administration may be necessary to demonstrate efficacy.

During many of the above trials, a number of unpleasant dose-related side-effects have emerged which may seriously limit the use of lodoxamide, particularly by the oral route, even if it proves to be efficacious. These effects, some of which have been noted on administration of the benzodipyran derivative (probicromil, see p. 16), include the production of sensations of body warmth (flushing), urethral burning, nausea, headache and, occasionally, vomiting [276, 277, 279, 282, 284, 285]. Increases in blood pressure have also been observed after oral lodoxamide ethyl [276]. The pharmacological mechanism of these effects has not been elucidated, though cholinergic stimulation [268] and release of catecholamines have both been suggested [277].

A number of *N*-troponyloxamate derivatives (96; Ayerst) were synthesized and investigated using passive paw anaphylaxis in the rat [286]. In this test, inhibition of oedema rather than of dye leakage (as in rat PCA) is used to determine antiallergic activity. Generally, substituents in positions 3 and 7

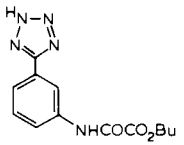


(96)

reduced activity; only the dioxamic ester (96, R = 5-$NHCOCO_2Et$) and the methoxy derivative (96, R = 6-OMe) were of comparable potency to the unsubstituted compound. Alternative esters of the parent compound were also investigated; bulky alkyl esters and the phenyl ester showed a slight reduction in oral activity. Replacing the oxamate NH function by sulphur or oxygen led to totally inactive compounds. The parent compound (96, R = H; AY-25,674) has been studied further [287].

Inhibition of passive peritoneal anaphylaxis [288] and passive lung anaphylaxis [289] have been demonstrated in the rat, but clinical studies were not undertaken following adverse animal toxicity studies [288].

The antiallergic properties of the tetrazolylphenyloxamate (97; MTB; Wakamoto) [290], 3-methoxyphenyl-4-thiazolyl-2-oxamate (98; F-1865; Pierre Fabre) [291, 292] and the coumarino-oxamic acid (99; CGP-13143; Ciba-Geigy) [293, 294] have also been described. All three compounds were orally



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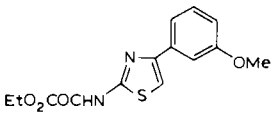

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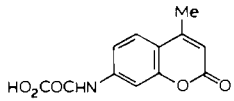
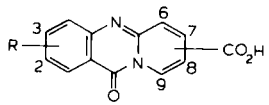


(99)

active in rat PCA and the last compound (99) was a potent inhibitor of active lung anaphylaxis in the rat [293].

NITROGEN BRIDGEHEAD COMPOUNDS

In recent years, a number of heterocycles which contain a nitrogen atom at a ring junction have been shown to possess antiallergic properties. Many of these compounds have structures which are virtually superimposable upon the well-known pharmacophores such as xanthone-2-carboxylic acid and 3-tetrazolylchromone.

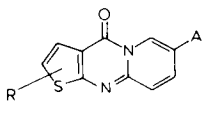


(100)

Several papers describing such compounds have been published by the Warner Lambert/Parke-Davis group. Of a series of pyrido[2,1-*b*]quinazoline derivatives (100), the 8-carboxy derivative (100, 8-CO_2H, R = H) and the corresponding linear benzo-fused analogue had significant activity in rat PCA by the intravenous route [295]. The compounds were shown *not* to be antihistamines, and the former compound was orally active. This led to the synthesis of substituted pyridoquinazolines (100, 8-CO_2H) [296]. The most active compound (i.v.) in rat PCA possessed 2- and/or 3-electron-donating

substituents, particularly alkoxy, and by the oral route, compounds (100, 8-CO_2H, R = 2-OMe and 2-Me) were the most active. The latter compound was found to be 10-times more potent orally than doxantrazole in preventing death in a rat allergic bronchospasm model, but only half as potent as an inhibitor of anaphylactic histamine release from rat peritoneal mast cells. Interestingly, compounds (100) and the corresponding tetrazoles inhibited human alkaline phosphatase (ALP), a membranal enzyme associated with calcium uptake in certain tissues, and ALP inhibitory potency paralleled rat PCA inhibitory activity [297].

The same company has reported activity in rat PCA for the closely related systems pyridothienopyrimidines (101, A = CO_2H, tetrazole or *N*-tetrazolylcarboxamide) [298], pyridazinoquinazolines (102, R^1 or R^2 = carboxylate)



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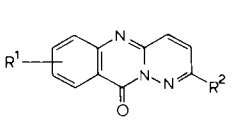

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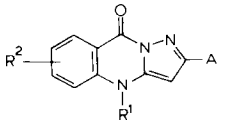


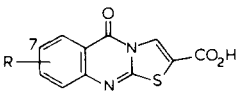
(103)

[299] and pyrazoloquinazolines (103, A = CO_2H, tetrazole or *N*-tetrazolylcarboxamide) [300]. Some members of each of these series showed oral activity in rat PCA.

Hoffman-La Roche also reported activity in rat PCA for compounds (100) and derivatives in which the carboxylic acid function was transferred to the benzo-ring [301]. The most potent compounds (i.p.) were the isopropyl derivative (100, 8-CO_2H, R = 2-isopropyl) and the isomer in which the two substituents had been interchanged; the latter compound was 10-times more potent than the former by the oral route (ID_{50} value = 0.026 mg/kg) and is one of the most potent compounds yet reported in rat PCA after oral administration. A compound also reported from Warner-Lambert/Parke Davis [296] (100, 8-CO_2H, R = 2-OMe), has been the subject of further biological study at Hoffman-La Roche Laboratories as Ro 21-7634. It was shown to be an orally active inhibitor of antigen-induced bronchoconstriction in passively sensitized rats and of rat passive peritoneal anaphylaxis [302]. Antigen-induced release of histamine from passively sensitized (IgE) rat peritoneal cells was inhibited by Ro 21-7634, as was histamine release induced by compound 48/80 and concanavalin A (but not by calcium ionophores). In these respects, Ro 21-7634 behaved like sodium cromoglycate, but, unlike sodium cromoglycate, it inhibited antigen-induced histamine and SRS-A release from actively sensitized guinea-

pig lung fragments, an IgG_1-mediated process [303]. The compound also stimulated phosphorylation of the 78,000 dalton protein in rat peritoneal mast cells and in this respect was 100-times as potent as sodium cromoglycate [304]. Human pharmacokinetic studies have also been undertaken [305]. After oral administration, the terminal plasma half-life varied from 2.3 to 21 h and approximately 50% of the dose was excreted unchanged. Doses of 1–50 mg were used and no adverse effects were noted; 98–99% of the drug was bound to plasma protein.

Thiazolo[2,3-*b*]quinazoline-2-carboxylic acids (104) described by workers at Hoffman-La Roche had potent oral activity in rat PCA. The most potent compounds by this route were (104, R = 7-OMe, 7-iPr and 7-SMe) [306]. Structures related to (104) in which the carboxylic acid function and the substituents R have been interchanged were also described [306] and similar structures have also been patented (Boehringer-Sohn) [307].



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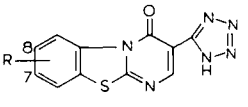

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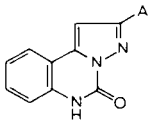


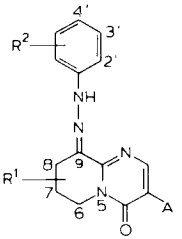
(106)

Several 3-tetrazolylpyrimido[2,1-*b*]benzothiazolones (105, Bristol-Myers) have been evaluated in rat PCA [308]. The parent system and the 8-chloro derivative (105, R = H and 8-Cl) were potent by the oral route, but other derivatives were considerably less active. The 7-chloro compound, for example, was 400-times less potent than its isomer. The two most interesting compounds were evaluated for bronchodilator activity. When administered intraduodenally they were shown to partially inhibit methacholine-induced bronchospasm in rats. In this respect they were quite potent but had poor efficacy.

The hydroxymethyl substituted pyrazolo[1,5-*c*]quinazoline (106, A = CH_2OH; SQ 13,847; pirquinozol; Squibb) has been shown to be an orally effective prodrug of the corresponding acid (106, A = CO_2H; SQ 12,903) [309]. SQ 13,847 inhibited rat PCA by the oral route (ID_{50} value $\approx$ 3 mg/kg). Like sodium cromoglycate, it was inactive in IgE-mediated PCA in the mouse and IgG_1-mediated PCA in the guinea-pig [310]. Pirquinozol, which is rapidly oxidized *in vivo* to the carboxylic acid in the rat [311], was inactive against rat PCA when co-administered with antigen (i.v.), whereas administration of the compound as little as 1 min before challenge was sufficient to produce inhibition [310]. When the acid (SQ 12,903) was dosed i.v. at challenge it was more than

160-times as potent as SQ 13,847. Against histamine release from rat peritoneal mast cells *in vitro*, SQ 12,903 was more than 100-times as potent as the hydroxymethyl compound [312]. These studies demonstrate that SQ 13,847 is acting as a latentiated derivative of SQ 12,903 (compare with the 3-hydroxymethylchromone (28)).

Nearly 70 hydrazonopyrido[1,2-*a*]pyrimidinones (107; Chinoin; UCB) were tested in rat PCA and also *in vitro* against histamine release from rat mast cells [313]. Compounds such as esters, amides and nitriles, in which the substituent A was not carboxylic acid, were essentially inactive. The most potent compounds contained a methyl substituent in position 6 (107, R^1 = 6-Me). Some of the racemates (107, R^1 = 6-Me, R^2 = H, 4′-OH, 4′-OEt and 2′-CO_2H) were resolved and in all cases the 6*S* enantiomer was shown



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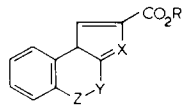

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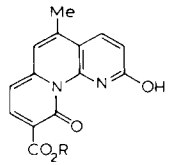
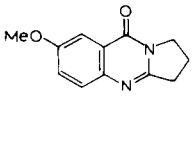


(109)

to be responsible for activity in rat PCA. For example, the (−)-enantiomer of (107, A = CO_2H, R^1 = 6-Me, R^2 = H) was 180-times less potent than the (+)-isomer (Chinoin 1045). This difference was not so marked *in vitro*. Chinoin 1045 was selected for further pharmacological [314] and clinical investigations. It was orally active in the rat against PCA, passive peritoneal anaphylaxis and passive anaphylactic bronchospasm [314].

Compounds of general structure (108; Roussel) are claimed to possess antiallergic properties [315, 316]. Weak activity in rat PCA was displayed by pyridonaphthyridines (109, R = H and Et) by the intraperitoneal route [317]. In addition, the pyrroloquinazoline [318] (110; compound 73/602; Central Drug Research, India) and the imidazopurinone [319, 320] (111; MJ 12504; Mead Johnson) and analogues [320] have been claimed to have antiallergic activity. The biological profile of the latter compounds probably corresponds more to that of theophylline than sodium cromoglycate, as they are phosphodiesterase inhibitors and bronchodilators. Compound (111) was 25-times



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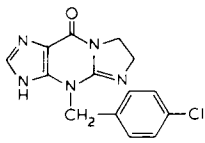

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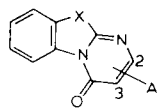


(112)

as potent as theophylline against allergen-induced bronchospasm in the rat, and of longer duration of action [320].

Many of the structures (112, A = CO_2H, tetrazole or *N*-tetrazolylcarboxamide; Riker) displayed activity comparable to sodium cromoglycate in rat PCA and some were orally active [321]. Generally, compounds with an acidic function in the 3-position were more potent and the most active compounds parenterally were benzimidazole derivatives (X = NMe), one of which (112, X = NMe, A = 3-CHN_4) was also most active by the oral route. The corresponding benzoxazole (112, X = O, A = 3-CHN_4) also had considerable oral activity.

MISCELLANEOUS ANTIALLERGY COMPOUNDS

A variety of other structures (113)–(130) have also been claimed to possess antiallergic activity. These are summarized in *Table 1.4*.

Table 1.4. MISCELLANEOUS COMPOUNDS WITH CLAIMED ANTIALLERGY ACTIVITY

Chemical class	*Structure [code number, name]*	*Company*	*Ref.*	*Biological tests in which activity was shown*
Naphthoquinone	(113)	Beecham's	322	rat PCA (moderate, some orally active)
Naphthoquinone	(114)	Beecham's	323	most potent compound i.v. in rat PCA had R = 6,7-dimethyl[a] cf. nitroindandiones [217]
Pyrazole	(115) [LC-6]	Cosmos	325, 326	rat PCA (low potency but long duration by the oral route)
Anthranilic acid derivative	(116) [AB-50]	Chugai	327	rat PCA and guinea-pig (IgG_1) PCA. OAc groups in AB50 converted to OH (AB23) after oral administration

(continued)

Table 1.4. continued

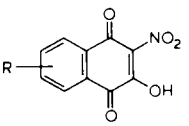
Chemical class	*Structure [code number, name]*	*Company*	*Ref.*	*Biological tests in which activity was shown*
Benzoxepine	(117)	Upjohn	328	some derivatives orally active in rat PCA when given 1 or 2 h before challenge
Phenylacetic acid	(118) [GPA-2476]	Ciba--Geigy	329	guinea-pigs and *Ascaris*-sensitive dogs; inactive in the clinic
Isoquinoline	(119)	USV/ Revlon	330	rat PCA, passively sensitized guinea-pig lung slices, phosphodiesterase inhibition
Benzoxazole	(120) [RHC 2871]	USV/ Revlon	331	rat mast cells (lack of cross-tachyphylaxis with sodium cromoglycate; irreversible inhibition); human basophils; dissimilar to sodium cromoglycate
	(121) [RHC 3024]	USV/ Revlon	332	rat mast cells (cross-tachyphylaxis with sodium cromoglycate – reversible inhibition); no effect on guinea-pig lung or human basophil models. Similar to sodium cromoglycate
Ether	(122) [W307, chlorphenesin]	Wallace	333	guinea-pig PCA; human leucocytes
Thioether	(123) [W2719]	Wallace	334	guinea-pig PCA; rat mast cells; rabbit leucocytes. Adverse animal toxicity; clinical testing abandoned
Anisamide	(124) [cloxacepride]	Merckle	335, 336	rat mast cells; may be a calmodulin antagonist
Pyrone Pyridine Pyrimidine	(125) (126) (127)	Tanabe	337	rat PCA (oral activity)
Pyrimidinylurea	(128) [WIN-40,882]	Sterling--Winthrop	338	human leukocytes; WIN-40,882 and analogues orally active in rat PCA [339]
Tetrahydrocarbazole	(129) [WIN-34,284, oxarbazole]	Sterling--Winthrop	340	SRS-A and bradykinin induced bronchoconstriction in guinea-pigs; active orally against human antigen challenge [341] and in seasonal allergic rhinitis [342]

(continued)

Table 1.4. continued

Chemical class	*Structure [code number, name]*	*Company*	*Ref.*	*Biological tests in which activity was shown*
Cyclohexane-carboxylic acid	(130) [IG-10, loxanast]	Hisamitsu/ Otsuka	343, 344	various immediate and delayed hypersensitivity reactions; preliminary clinical trials in asthma and atopic dermatitis reported to have produced good results [345]

[a] During the preparation of this manuscript, the detailed pharmacology of this compound was published [324].



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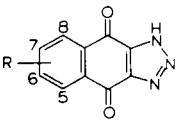

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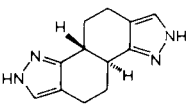

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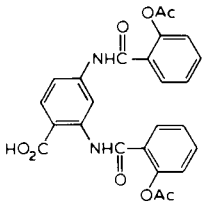

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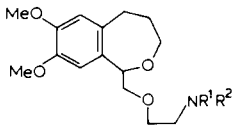

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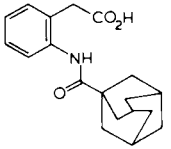

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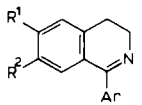

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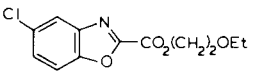

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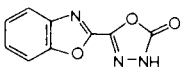

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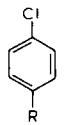
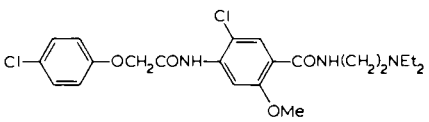


(122) R = $-OCH_2CH(OH)CH_2OH$

(123) R = $-S(CH_2)_4OH$



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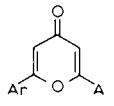

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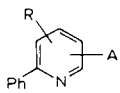

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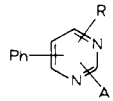
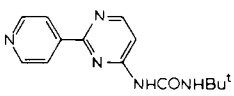


(127)

A = tetrazole or N–tetrazolylcarboxamide



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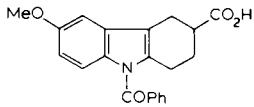

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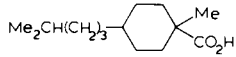

(130)

CONCLUSIONS

The variety of chemical structures and companies which has been referred to in the previous section bears witness to the considerable effort which has been generated in the search for therapeutic substances of the sodium cromoglycate type. The lack of progress in producing marketable entities is quite remarkable.

Some compounds have emerged which showed initial promise, but which have since been discarded (e.g., doxantrazole, nivimedone, bufrolin, proxicromil, probicromil), while others are, at the time of writing this review, still progressing along the difficult path towards the market-place (e.g., lodoxamide, zaprinast, Wy 41195, nedocromil, minocromil, sudexanox). However, other compounds which are claimed to have mast-cell-stabilizing properties, but whose primary mode of action is that of antagonizing histamine at H_1 receptors, have also become available for therapeutic use (i.e., ketotifen and oxatomide).

Problems in this field of research have been referred to and consist essentially of an incomplete knowledge of the mode of action of sodium cromoglycate, the lack of predictability of activity in animal or *in vitro* models and in human provocation tests for therapeutic activity, and the difficulties inherent in therapeutic trials in such a variable disease as asthma. The future will see further attempts to improve our knowledge and our testing and trial procedures.

We still believe that it is worthwhile developing other compounds of the sodium cromoglycate type for the prophylactic treatment of asthma. Although the consumer might prefer a formulation to be taken by mouth, it is also our belief that formulations, either in powder or aerosol form, to be administered by inhalation directly to the lungs will continue to have a place.

This review has concentrated on attempts to develop compounds of a specific type to be used in the treatment of asthma and associated diseases. It is also important to remember the contribution made by other classes of drugs in this therapeutic area, namely, the bronchodilators (β_2-adrenoreceptor stimulants, phosphodiesterase inhibitors), mediator antagonists (antihistamines, anticholinergics) and corticosteroids.

The drug therapy of asthma and related diseases has been greatly improved during the past 20 years. Future years hold out the promise of improving the life of the asthmatic even further, although, as can be gauged from this review, progress to date in the development of prophylactic antiallergy agents, following the discovery of sodium cromoglycate, has been slower than might have been expected.

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2 Polypeptides from Snake Venoms which act on Nerve and Muscle

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GENERAL INTRODUCTION

Although there is some debate on the most suitable systematic classification of the snakes, most would agree that a given animal can be assigned to one of between 11 and 13 families. Venomous snakes, however, are represented in only five families: the Colubridae (colubrids), Elapidae (elapids), Hydrophiidae (sea-snakes), Viperidae (true vipers) and Crotalidae (pit-vipers). All snakes of the latter four families are venomous, whereas of the colubrids, only a few are venomous.

The evolution of the ability to manufacture and store a venom, and the structural modifications to the jaw and teeth that allow the delivery of the venom, are of major significance. For the reptile concerned, they allow relatively large prey to be immobilized prior to digestion and they initiate digestion. For man, the consequences are reflected in the number of victims hurt, maimed and even killed by snake bite [1]. Fortunately, venomous snakes probably deliver venom on only a minority of occasions, since there is good evidence that most, if not all, can deliver defensive bites that do not result in the inoculation of venom [2].

An envenoming bite by a snake may give rise to a diversity of symptoms, including local necrosis, neuromuscular weakness, coagulation defects and cardiovascular irregularities, but it is generally considered that venoms can be differentiated into those which act primarily on the 'nervous system' and those which act to disturb the 'cardiovascular system'. This differentation is considered to reflect the activity of the predominant toxic fraction(s) in the venom; but it is important to acknowledge that almost all snake venoms contain toxins that can cause temporary or more long-lasting neurological changes, cardiovascular problems and soft-tissue necrosis [3–9].

The isolation and characterization of individual toxic fractions from crude venoms is a relatively new activity, and as recently as 1965 it was reported that

"no venom has yet been comprehensively fractionated and there is no one procedure that gives a complete separation of the toxic fractions and enzymes" [10]. It seems that the drive to purify the toxic fractions depended not simply on an inherent scientific interest in animal venoms and the development of the appropriate technology, but on the observation that certain toxins could be used to define specific biological systems, such as the acetylcholine receptor and its distribution in skeletal muscle, and the cascade of reactions leading to the coagulation of blood [11,12].

This review is concerned only with snake venom toxins that act on nerve and muscle. Where primary structures of the toxins are given, the single-letter code is used to identify the constituent amino acids. Elsewhere the three-letter abbreviation is employed. Both conventions are presented in *Table 2.1.*

Table 2.1. ABBREVIATIONS FOR AMINO ACIDS

Amino acid	*Three-letter abbreviation*	*One-letter symbol*	*Amino acid*	*Three-letter abbreviation*	*One-letter symbol*
Alanine	Ala	A	Histidine	His	H
Arginine	Arg	R	Isoleucine	Ile	I
Asparagine	Asn	N	Leucine	Leu	L
Aspartic acid	Asp	D	Lysine	Lys	K
Asparagine or			Methionine	Met	M
aspartic acid	Asx	B	Phenylalanine	Phe	F
Cysteine	Cys	C	Proline	Pro	P
Glutamine	Gln	Q	Serine	Ser	S
Glutamic acid	Glu	E	Threonine	Thr	T
Glutamine or			Tryptophan	Trp	W
glutamic acid	Glx	Z	Tyrosine	Tyr	Y
Glycine	Gly	G	Valine	Val	V

CLASSIFICATION OF THE TOXINS

Toxins acting on nerve and muscle may be differentiated into one of the following four categories:

(1) The postsynaptically active neurotoxins. These bind to the receptors on the postsynaptic membrane to produce neuroeffector blockade.

(2) The presynaptically active neurotoxins. These inhibit or potentiate transmitter release from motor nerve terminals.
(3) The myotoxic toxins. These directly damage nerve and muscle.
(4) The cytotoxic toxins. These are general cytotoxins which incidentally damage nerve and muscle.

These categories are rather loosely formed and to some extent overlap. For example, although toxins blocking neuroeffector transmission tend to be specific in their activity, many presynaptically active neurotoxins not only inhibit transmitter release but also destroy motor nerve terminals and skeletal muscle. The four categories are considered in turn.

POSTSYNAPTICALLY ACTIVE NEUROTOXINS

INTRODUCTION

By far the majority of toxins included in this category are the so-called curarimimetic toxins that block neuromuscular transmission at the skeletal neuromuscular junction. To date, about 60 such toxins have been isolated. All of the toxins are from the venoms of snakes of families Elapidae and Hydrophiidae, and most venoms yield several closely related but distinct toxins. *Dendroaspis viridis* (the Western green mamba), for example, elaborates at least four postsynaptically active neurotoxins, and the sea-snake *Laticauda semifasciata* elaborates a similar number.

STRUCTURE

The toxins may be conveniently assigned to one of two groups, the short and the long neurotoxins. The short neurotoxins contain between 60 and 62 residues arranged in a single chain and cross-linked by four disulphide bridges. Approximately 30 such toxins have so far been identified in the venoms of 20 or so different species of families Elapidae and Hydrophiidae. The long neurotoxins contain between 69 and 74 residues arranged in a single chain and cross-linked by five disulphide bridges. Approximately 30 long toxins have been identified in the venoms of 15 or so different species. The only exceptional toxin so far identified is toxin LsIII, isolated from *Laticauda semifasciata*, which has 66 residues in a single chain and five cross-bridges (see *Figure 2.1*).

The postsynaptically active neurotoxins form a closely related homologous series. They possess a number of invariant or conservative residues which include Tyr-25, Trp-29, Asp-31, Arg-37, Gly-38, Gly-44, Gly-56 and Pro-50

(homology positions). In structural terms, the most important residues are the half-cystines. In both the short and the long neurotoxins, these are located at homology positions 3, 17, 24, 45, 49, 61, 62 and 68; in the long toxins the additional pair of half-cystines is located at positions 30 and 34. Cross-bridging takes place between Cys-3 and Cys-24, Cys-17 and Cys-45, Cys-30 and Cys-34 (where present), Cys-49 and Cys-61 and Cys-62 and Cys-68. The sequences of a number of the long and short toxins, arranged in such a way as to emphasize the homologous nature of the toxins, are illustrated in *Figure 2.1.*

Relatively few of the toxins have been subject to detailed three-dimensional analysis, but so closely related are these structures that it is likely that the crystal structures of the short neurotoxins are adequately described by that assigned to erabutoxin b, isolated from the venom of *Laticauda semifasciata* [13,14] and that of the long toxins to that assigned to toxin 3 (i.e., α-cobratoxin) isolated from the venom of the Siamese cobra *Naja naja siamensis* [15]. The structures

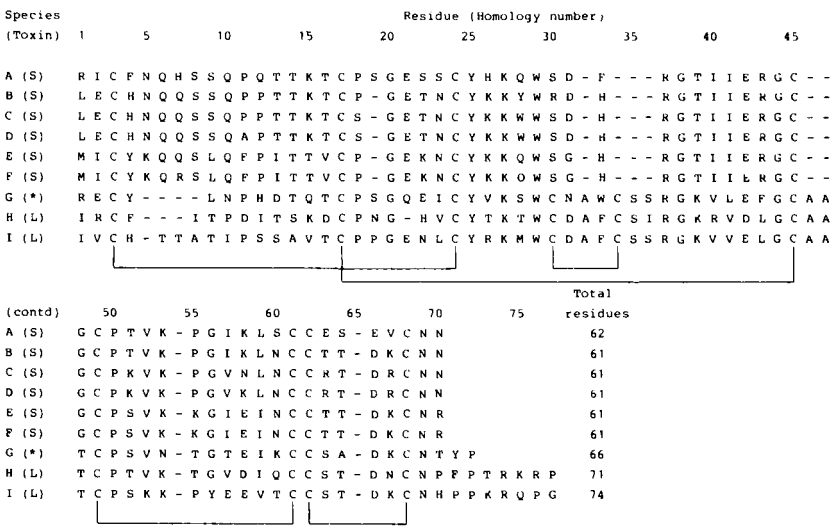

Figure 2.1. Sequences of selected short (S) and long (L) toxins. The bars link the half-cystine residues involved in the formation of disulphide bridges. (A) Laticauda semifasciata *(erabutoxin b); (B)* Naja nigricollis *(toxin α); (C)* Naja naja oxiana *(toxin 2); (D)* Naja haje philippinensis *(short toxin); (E)* Naja haje annulifera *(toxin CM10); (F)* Naja haje annulifera *(toxin CM12); (G)* Laticauda semifasciata *(toxin LsIII); (H)* Naja naja siamensis *(toxin 3); (I)* Bungarus multicinctus *(α-bungarotoxin).* **LsIII is anomalous (see text).*

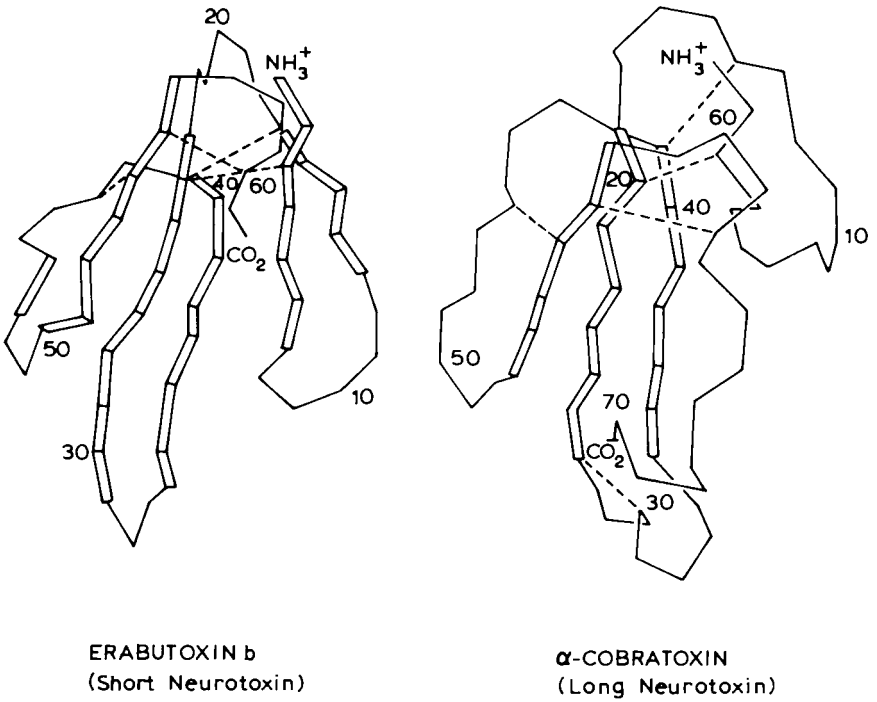

Figure 2.2. Peptide backbones of typical short and long neurotoxins. β-pleated sheet formations are indicated by 'plates' and disulphide bridges by broken lines. The structures are slightly modified from Hider and Dufton [48].

of crystalline erabutoxin b and toxin 3 are illustrated in *Figure 2.2*. Both are arranged in the general form of three loops plus a tail. Residues 1–6, 13–16, 23–30, 34–40 and 50–55 in the short toxins and 20–25, 36–40 and 51–57 in the long toxins are regions of antiparallel β-pleated sheet conformations; no α-helical conformations occur in these toxins.

PHARMACOLOGICAL PROPERTIES

Both the short and the long toxins bind specifically to the postsynaptic nicotinic cholinergic receptors at the skeletal neuromuscular junction, and thus prevent the interaction between acetylcholine (ACh) released from motor nerve terminals and the receptors. The degree of the resulting inhibition of transmission is a function of receptor occupancy. Binding is not associated with

either muscle fibre depolarization or with a reduction in muscle excitability [16], and nerve terminal spikes can be recorded even after total neuromuscular blockade has been achieved [17].

Following surgical denervation, ACh receptors appear in the extrajunctional region of the membrane [18]. These receptors also act as binding sites for the toxins and, as with innervated muscle, binding is accompanied neither by depolarization nor by any loss of direct excitability in the muscle [19]. Binding to both junctional and extrajunctional receptors is saturable, dependent upon toxin concentration, but relatively insensitive to changes in temperature [20]. Agents known to desensitize receptors inhibit toxin binding [20–22].

Whether toxins bind to nicotinic cholinergic receptors in autonomic ganglia is still to be fully resolved. Consistent experimental evidence for the blockade of neuroeffector transmission is lacking. Brown and Fumagali [23], for example, claim that the long toxin α-bungarotoxin, isolated from the venom of the banded krait *Bungarus multicinctus*, does not block transmission in rat superior cervical ganglion, while Dun and Karczmar [24] claim that the effect of the transmitter (ACh) is inhibited in the same preparation by α-bungarotoxin. There is, however, clear evidence of binding in a variety of tissues, including rat superior cervical ganglion and chick cervical ganglion [25,26]. The 'classical' test of binding to a structure as well characterized as a cholinergic receptor is to study the interaction between the ligand under investigation and known receptor agonists and antagonists. Even this approach has been unsuccessful in clarifying the question of whether the binding site is the cholinergic receptor involved in neuroeffector transmission (see Ref. 27, for example) and one is led to conclude that binding is probably neither to the receptor itself, nor to the ionophore associated with the receptor, but to an unidentified structure.

The long toxin α-bungarotoxin also binds to structures in brain homogenates, in synaptosomes and in cholinergically innervated areas of the brain [28,29], but functional disruption of CNS function has been documented only in the optic tectum, where retino-tectal transmission is blocked [30]. This is the only central site where there is a possible correlation between binding and the disruption of physiological responses.

Several studies have been made in an attempt to identify the α-bungarotoxin-binding site in the CNS, usually by comparing its characteristics with those of so-called nicotinic cholinergic receptors. A recent investigation showed that the binding site shared no common antigenicity with a number of peripheral cholinergic receptors [31]. This is not particularly helpful though, because there is no evidence that peripheral and central nicotinic cholinergic receptors share such determinants. As with ganglionic binding sites, the issue awaits clarification.

A potentially useful contribution to this endeavour has been made by Ono and Salvaterra [32], who observed that the long toxins α-bungarotoxin and *Naja naja siamensis* toxin 3 (*Figure 2.1*) bind to a nicotinic cholinergic site in homogenates of *Aplysia californica* ganglia. In intact ganglia, both toxins reversibly block the increase in chloride conductance (gCl^-) caused by the application of ACh, but not the increase in sodium (gNa^+) or potassium (gK^+) conductance. Moreover, the increase in gCl^- mediated by a variety of non-cholinergic agonists such as histamine and glutamate is also blocked. Since the respective responses to ACh (that is, increase in gCl^-, increase in gNa^+ and increase in gK^+) are mediated by three distinct receptor systems [33], the results suggest that the long toxins are capable of highly selective binding to only a proportion of the total population of nominally cholinergic receptors. Moreover, this population of accessible receptors may be linked to a Cl^- ionophore that is usually stimulated by non-cholinergic agonists. By extrapolation, it may be that in parts of the vertebrate CNS, and perhaps in autonomic ganglia, there exists a population of receptors, normally activated by a non-cholinergic agonist, which acts as the α-bungarotoxin-binding site. This would satisfy two conditions: the presence of a binding site and the lack of a correlation between toxin binding and apparent physiological function.

Several workers have reported that the postsynaptic toxins are inactive at sites using muscarinic cholinergic receptors (Ref. 16, for example), but binding studies do not appear to have been performed.

It is generally assumed that the toxins inhibit transmission at the neuromuscular junction by simple receptor blockade. It has been reported, however, that α-bungarotoxin and *Naja naja siamensis* toxin 3 (*Figure 2.1*) can initiate, or accelerate, the rate of receptor desensitization in skeletal muscle [34]. Since desensitization occurs when the toxins are applied to the cell interior, as well as when applied to the cell exterior, it would seem the toxins stimulate desensitization indirectly by acting on a 'non-receptor' structure. It is known that the postsynaptic toxins can be internalized by the process of endocytosis [35], and it seems possible that the toxins effect blockade by a combination of receptor blockade – by its nature an extracellular phenomenon – and receptor desensitization, possibly following internalization.

One puzzling feature of the interaction between toxins and the cholinergic receptor of skeletal muscle is that, despite having a dissociation constant of the order of 0.1 nM, the kinetics of association and dissociation are very slow. The short toxins tend to be faster in both respects than the long toxins, but the dissociation in general is so slow that the toxins may be considered virtually irreversible [36–38]. Perhaps the act of binding requires the induction of a slight conformational change in the toxins for completion.

STRUCTURE-ACTIVITY RELATIONSHIPS

If a profile of erabutoxin b is prepared, it is seen to represent a 'saucer with a footed stand' [39]. The most significant feature, however, is that the four cross-bridges form the core of the structure, and so it is not surprising that the reduction of these bonds results in the loss of pharmacological activity as well as structural organization [40]. The stand is formed by the terminal residues. The invariant and conservative residues are almost all found between positions 32 and 49 (homology positions 33–56), which would indicate that this region is intimately involved in receptor binding.

Detailed studies on the major 'active region' of the postsynaptic toxins have been made using three quite distinct approaches. The first involves neutralizing the charge on selected residues and determining the consequences on affinity and toxicity, the second is concerned with studying the effects of selective derivatization on binding, and the third involves the preparation of synthetic polypeptides and measuring their capacity to bind to the cholinergic receptor.

The neutralization of the positive charge on Lys-27 and Lys-47 (homology positions 27 and 53), of the short toxin *Naja nigricollis* α-toxin (*Figure 2.1*) results in a marked reduction in affinity for ACh receptor-rich membranes without any change in the CD spectrum of the toxin, whereas neutralization of Lys-15 (homology position 15) and Lys-51 (homology position 53) has little effect on affinity [41]. This observation implies that the reduction in affinity consequent upon the neutralization of the charge on Lys-27 and -53 (homology positions) is not a direct consequence of a conformational change. Significantly, the change in affinity is not total, and this could mean that Lys-27 and Lys-53 (homology positions) are specifically involved in binding, but are not uniquely required for binding.

Similar results pointing to the importance of Lys-27 and -53 (homology positions) have been obtained by Russian workers [42] who examined the accessibility of spin-labelled and fluorescent residues in the short toxin *Naja naja oxiana* toxin 2 (*Figure 2.1*) when complexed with solubilized receptor from *Torpedo* electric organs. By contrast, the derivatization of Trp-29 (homology position 29) with nitrophenyl-sulphonyl chloride reduces the affinity of the toxin for the receptor, but formylation has little real effect [41]. This would indicate that Trp-29 is not uniquely involved in binding; indeed, it may not even be directly involved. It may be that the introduction of a bulky derivative disrupts the organization of the toxin, perhaps by causing a separation of the strands defined by residues 25–32 and 55–60 (homology positions).

For some residues the situation is even less clear. Asp-31 (homology position) is invariant, and deviant toxins (*Naja haje annulifera* toxins CM-10

(*Figure 2.1*) and CM-12 (*Figure 2.1*), and *Laticauda semifasciata* LsIII (*Figure 2.1*), tend to be of low toxicity, but derivatization of Asp–31 does not necessarily reduce toxicity [43]. Likewise, it has been reported that the accessibility of Leu-1, Glu-2, Lys-15, Lys-26 and His-33 (homology positions) is reduced following binding of *Naja naja oxiana* toxin 2 to receptor, and yet none of these residues appears to be directly involved in binding [42].

A more recent technique of determining the 'active centre' of the postsynaptic neurotoxins has been developed in which synthetic reproductions of the region 16–48 (homology positions 16–55) of the major short toxin of *Naja naja philippinensis* (*Figure 2.1*) were prepared [44]. The reproductions were cyclized by oxidation, and two peptides, A and B were isolated (Juillerat and Bargetzi, 1980 quoted in Ref. 44). The binding of the peptides to receptor protein isolated from the electroplax of *Torpedo* was compared with the binding of α-bungarotoxin. Peptide B bound with high affinity (2.2×10^{-7} M), and although this is much lower than the affinity of the native toxin ($(3.0–4.0) \times 10^{-10}$ M), it was sufficiently high to allow the conclusion that the region was probably closely associated with the active centre. Perhaps the most interesting possibility is that the synthesis of 'active analogues' followed by affinity and conformation studies could yield a great deal of information on structure-activity relationships.

One problem associated with determining the precise binding site concerns the organization of the toxins when they bind to the receptor. Most information has been derived from the X-ray diffraction analysis of crystals of the toxins. Crystallization of these molecules is not easy, and usually demands high salt concentrations, low pH and the use of organic solvents such as isopropanol. Since there is strong evidence from studies of NMR and CD spectra that the structure of the toxins in solution varies with temperature, pH and the presence of organic solvents such as isopropanol [45–47], it seems possible that the structure of the toxins in aqueous solutions of neutral pH differs from that determined when the toxin has been crystallized from an acidic organic solution. Moreover, the crystal structures may be anomalous in that they do not reflect the possible flexibility of the molecules. It has been suggested [48] that there are two possible conformations for residues 30–37 (homology positions) in the short and long toxins, and that these two conformations reach equilibrium (*Figure 2.3*). In configuration 3a, the relationship between Asp-31 and Arg-33 (homology positions 31 and 37) is similar to that proposed by Low, and which is considered to relate to the structure of ACh, and in configuration 3b the relationship is claimed to resemble the curare-like alkaloids, in which two aromatic nuclei, two positive charges and two hydrogen bond acceptors are in close apposition. A similar model has been proposed by other workers [41].

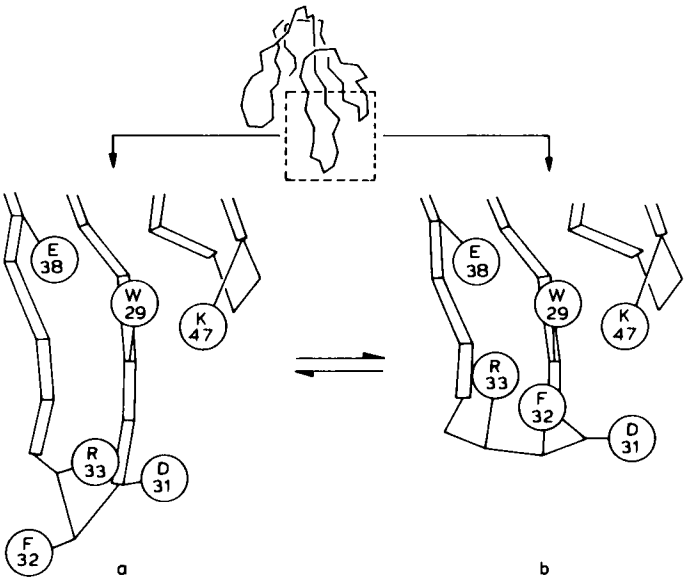

Figure 2.3. Short neurotoxins may exist in two conformations which reach equilibrium (a and b). Slightly modified from Hider and Dufton [48].

A single toxin molecule can bind up to four antibody molecules. Only one antibody molecule needs to bind to reduce the affinity of the toxin for cholinergic receptor [41]. This apparently paradoxical finding is close to resolution. A monoclonal antibody (M_{α}-1 raised against *Naja nigricollis* α-toxin) has been used to identify one antigenic site on the toxin. This site, nominated epitope 1, is topographically distinct from the receptor-binding site of the toxin. Epitope 1 does not involve all antigenic residues. Since these uninvolved residues can be grouped into three distinct regions, it would seem that there exist on any one toxin molecule four antigenic epitopes [49]. Two of these putative epitopes overlap the toxin binding site, and so antigens binding to these epitopes might be expected to reduce receptor binding by the toxin by 'simple' steric hindrance. Antigens binding to the other epitopes, including epitope 1, cannot act in this way because they do not overlap with the receptor-binding site. There can be only two explanations for the reduction in affinity of toxin for receptors when antigens bind to these latter epitopes. Either the

antigens induce a conformational change in the toxin such that it is no longer capable of recognizing the receptor, or the toxin-receptor complex is unstable.

The recognition that the postsynaptically active neurotoxins are flexible, and that they may possess several distinct antigenic regions as well as a binding site, is an exciting development, and the implications of the findings are discussed in detail in a recent review [50].

PRESYNAPTICALLY ACTIVE NEUROTOXINS

INTRODUCTION

By comparison with the 50–60 postsynaptically active neurotoxins so far isolated from snake venoms, approximately 12 with marked presynaptic activity have been identified (*Table 2.2*). Since they have been isolated from the venoms of snakes of families Elapidae, Viperidae and Crotalidae, it seems

Table 2.2. PRESYNAPTICALLY ACTIVE NEUROTOXINS

Toxin	*Venom source*		*Effect on transmitter release* [b]
	Species	*Family*	
Notexin	*Notechis scutatus*	Elapidae	inhibition
Notechis II-5	*Notechis scutatus*	Elapidae	inhibition
Taipoxin	*Oxyuranus scutellatus*	Elapidae	inhibition
III-A	*Bungarus caeruleus*	Elapidae	inhibition
III-B	*Bungarus caeruleus*	Elapidae	inhibition
β-Bungarotoxin [a]	*Bungarus multicinctus*	Elapidae	inhibition
Crotoxin	*Crotalus durissus terrificus*	Crotildae	inhibition
Caudoxin	*Bitis caudalis*	Viperidae	inhibition
Mojave-toxin	*Crotalus scutulatus scutulatus*	Crotalidae	inhibition
Neurotoxic complex	*Vipera ammodytes ammodytes*	Viperidae	inhibition
Dendrotoxin	*Dendroaspis augusticeps*	Elapidae	potentiation
Toxin I	*Dendroaspis polylepis*	Elapidae	potentiation

[a] Note that β-bungarotoxin is only one of several closely related toxins isolated from the venom of *Bungarus multicinctus*. Some authors indicate the precise toxin under investigation by use of a subscript (see Refs. 53, 83, for example). Where no subscript is used, it is probable that β_2-bungarotoxin (see Ref. 83) is used.

[b] From skeletal motor nerves of vertebrates.

probable that there are many more awaiting recognition. The majority of the presynaptically active neurotoxins inhibit transmitter release and they will be discussed at some length. The few toxins that enhance transmitter release have not yet been well characterized, and they will be discussed in a more abbreviated manner.

TOXINS THAT INHIBIT TRANSMITTER RELEASE

Structure

Although β-bungarotoxin was the first presynaptically active toxin to be isolated [16,51], the first of the toxins to be fully sequenced was notexin [52]. It is a basic polypeptide comprising 119 amino acids, organized in a single chain that is cross-linked by seven disulphide bridges.

A second presynaptically active toxin, notechis II-5, has been isolated from the venom of *Notechis scutatus*. It too is a basic polypeptide of 119 amino-acid residues arranged in a single chain and cross-linked by seven disulphide bridges [53].

All the other presynaptically active toxins are complexes of between two and four subunits. β-Bungarotoxin, for example, consists of two covalently linked subunits [54]. One subunit (β-bungarotoxin A) is a basic polypeptide of 120 amino acids in a single chain cross-linked by six disulphide bridges. The second subunit (β-bungarotoxin B) is a much smaller acidic polypeptide of 60 amino acids arranged in a single chain.

Taipoxin is a 1:1:1 ternary complex of three subunits, α-, β- and γ-taipoxin [55]. The subunits are not covalently linked, and can be separated at neutral or slightly acidic pH in the presence of 6 M guanidine hydrochloride, or in media of high ionic strength and low pH. The dissociation by guanidine is reversible, but acid dissociation is irreversible. α-Taipoxin is a strongly basic polypeptide of 119 amino-acid residues, arranged in a single chain and cross-linked by seven disulphide bridges. β-Taipoxin is a neutral polypeptide. It comprises 120 amino-acid residues, arranged in a single chain and cross-linked by seven disulphide bridges. This subunit probably exists in two iso-forms, designated β_1 and β_2. The iso-forms differ slightly (probably by a maximum of 12 residues) and appear to be completely interchangeable in the formation of the ternary complex. The γ-subunit is acidic and rather larger than both the α- and β-subunits. It is a single-chain sialoglycopolypeptide of 135 amino-acid residues cross-linked by eight disulphide bridges.

Dissociation of the ternary complex in media of high ionic strength and low pH results in an apparent reduction in the length of the γ-chain (as calculated

from elution characteristics from a Sepharose gel) but no change in amino-acid composition. It is postulated that this form of dissociation results in a disruption of the integrity of the γ-subunit, largely by removing the carbohydrate moiety, and that this results, in turn, in the irreversibility of the dissociation. It should be noted, however, that the precise nature of the ternary complex of taipoxin, and the forces that maintain it, are not fully understood.

Crotoxin [56] is a complex of two subunits. The subunits are not covalently linked and their dissociation is reversible [57]. The largest chain, crotoxin B (or CB), is a basic, single-chain polypeptide of 140 amino-acid residues, cross-linked by eight disulphide bridges. It may exist in more than one iso-form [58]. The smaller chain, crotapotin (crotoxin A, CA), is acidic and comprises a nominal 88 amino acids. It is, however, a complex of three much smaller chains (crotapotin A, B, C). Further, crotapotin A appears to exist in two iso-forms, designated A_1 and A_2 [59].

Crotapotin A contains 40 amino-acid residues, crotapotin B contains 34, and crotapotin C only 14; the three chains are bound together covalently and the complex contains seven disulphide bridges. Seven of the half-cystines are found in crotapotin A, five in crotapotin B and two in crotapotin C. The precise organization of the three chains is not known, but it seems clear that they are present in the complex in the molar ratio 1:1:1 [59]. As well as crotoxin B and crotapotin, the crotoxin complex may contain other fractions. These include crotamine, a small basic single-chain polypeptide of 42 amino-acid residues [60], an acidic phospholipase and 'Fraction III' [57], which possibly corresponds to 'crotactin' [61]. Not all samples of venom contain these trace constituents, and indeed crotamine is particularly interesting because its presence or absence may delineate different populations (or subspecies) of *Crotalus durissus terrificus* [62,63]. The highly variable nature of the crotoxin complex makes it difficult to investigate with confidence and may explain many seemingly anomalous aspects of its pharmacological behaviour (q.v.).

Mojave-toxin was isolated from the venom of the Mojave rattlesnake *Crotalus scutulatus*. Unlike crotoxin, it is strongly acidic (isoelectric point 4.7) and was originally classified as a 'cardiotoxin' [64]. The molecular weight of the toxin (approximately 22,000) was determined by column chromatography on Sephadex G-75, but since the apparent molecular weight fell to 12,000 after gel electrophoresis, it was considered probable that Mojave-toxin also consisted of more than one subunit [64]. A more detailed investigation confirmed that Mojave-toxin consisted of two subunits. One subunit, Mojave-toxin B, is strongly basic, and the other, Mojave-toxin A, is strongly acidic [65,66]. Neither the relative sizes of subunits A and B, nor their primary structures have yet been determined.

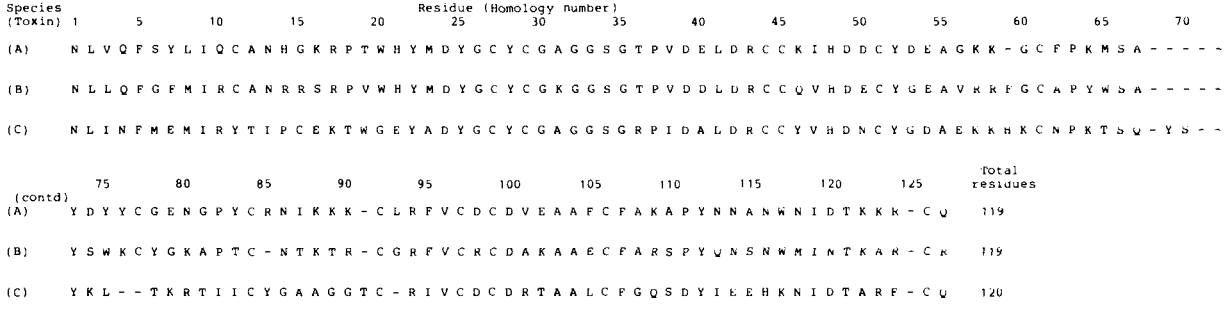

Figure 2.4. Sequences of selected presynaptically active toxins with PLA_2 activity. (A) Notechis scutatus *(notexin); (B)* Oxyuranus scutellatus *(taipoxin α-chain); (C)* Bungarus multicinctus *(β-bungarotoxin A-chain). Disulphide bridges are probably formed between half-cystines 11 and 77, 27 and 126, 29 and 45, 44 and 106, 51 and 99, 61 and 92, and 84 and 97 (see text).*

A neurotoxic complex of basic acidic polypeptides has been isolated from the venom of the Bulgarian viper, *Vipera ammodytes ammodytes* [67,68] and a basic PLA_2 with neurotoxic activity called caudoxin has been isolated from the venom of the horned adder *Bitis caudalis* [69,70].

The pharmacological properties of the various toxins will be discussed later, but at this point it is useful to consider that all are phospholipases, probably of the A_2 type. The single-chain toxins, notexin and notechis II-5, are active in this regard, but in the case of the complex toxins, usually only a single subunit possesses such activity. For example, α-taipoxin, chain A of β-bungarotoxin, and crotoxin B are the phospholipase components of taipoxin, β-bungarotoxin and crotoxin, respectively. In each case, the chain with phospholipase activity is the most toxic chain in the complex, and these chains are structurally homologous (*Figure 2.4*). The half-cystine residues are found in homology positions 11, 27, 29, 44, 45, 51, 61, 77, 84, 92, 97, 99, 106 and 126; chain A of β-bungarotoxin, which has six, rather than seven, disulphide bridges, is deficient in one pair of half-cystines (homology positions 11, 77) but contains one odd half-cystine at homology position 15 (*Figure 2.4*). There is no information on the three-dimensional structure of the 'active' chains, although crystals of notexin have been obtained [71] and details of the organization of notexin may be anticipated. Since the phospholipase chains are homologous with porcine pancreatic PLA_2 [72], it might be expected that a knowledge of the structure of the porcine enzyme would be a good guide to the structure of the toxins. Preparation of good crystals of the active form of the porcine enzyme has proved difficult, but bovine pancreatic PLA_2 has been crystallized [73]. The bovine enzyme is predominantly α-helix in organization, with a relatively modest β-pleated sheet component [73]. By contrast, crotoxin B is mainly β-pleated sheet [74]. Chain A of β-bungarotoxin may [75] or may not [54] be predominantly α-helix, and Mojave-toxin B is almost certainly mainly α-helix in configuration [55]. Without more information, it is difficult to know how reliable some of these data are, especially since the secondary structure of these toxic components appears to vary with pH and, significantly, with complex formation (see Ref. 74, for example).

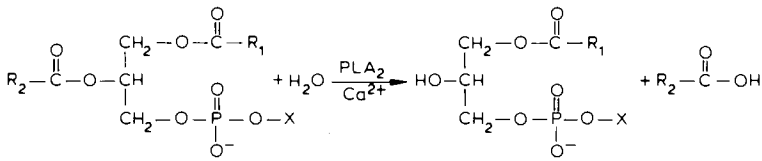

Figure 2.5. Hydrolysis of phospholipids by phospholipase A_2.

In one respect, however, the structure of the bovine enzyme probably is a reliable guide to the structure of the neurotoxic PLA_2 enzymes. The cross-bridges in the bovine pancreatic PLA_2 are known to occur between half-cystines at homology positions 11 and 77, 27 and 126, 29 and 45, 44 and 106, 51 and 99, 61 and 92, and 84 and 97, and it seems likely that this is the case in the toxins (see *Figure 2.4*).

Pharmacological properties

Phospholipase activity. The biochemistry and pharmacology of phospholipase A_2 enzymes (PLA_2) has been discussed at length in a number of recent reviews (see Refs. 76, 77, for example) and so only a summary will be provided here. Moreover, reference will be made only to work involving neurotoxic PLA_2. The enzymes hydrolyse the ester bond at the C_2 position of 3-*sn*-phosphoglycerides, liberating fatty acids and leaving as the reaction product the appropriate lysophosphatide (*Figure 2.5*). It is now universally accepted that Ca^{2+} is an essential cofactor, although some earlier reports suggested otherwise. The most likely explanation of this apparent discrepancy is that many substrates (and many enzyme preparations) are prepared in the presence of Ca^{2+}, and they retain sufficient Ca^{2+} for activation. When the experiments are done in the presence of EDTA or EGTA, activation may be abolished [76].

Most other divalent cations inhibit the Ca^{2+}-activated enzymes [78–80], although Mg^{2+} at low concentrations may stimulate the hydrolytic activity of some neurotoxic PLA_2 [79,80] and Sr^{2+} does not always inhibit activity when Ca^{2+} is present [79].

The neurotoxic PLA_2 enzymes can degrade substrate in a variety of forms, including highly ordered systems such as micelles. Membrane-bound lipids may also be hydrolysed [78,81].

Micelles are preferentially hydrolysed at their gel-liquid phase transition temperatures, and the rate of hydrolysis can be reduced by incorporating cholesterol into the micelles [82]. There is unanimous, and not unexpected, agreement that hydrolysis is stimulated by the presence of detergents such as deoxycholate [77,78,83]. The presence of detergent is often stated to be an absolute requirement of the neurotoxic enzymes [84], but it seems far more probable that it relates more closely to the organization of the substrate [85–87]. Indeed, notexin, for example, is perfectly able to hydrolyse micelles and membrane-bound phospholipids in the absence of detergent [78].

The neurotoxic PLA_2 enzymes are very stable, probably, as a result of the extensive cross-bridging. As a result, they are active over a wide range of pH

and temperature, and retain activity even when heated to temperatures in excess of 60° C [10,77].

The hydrolytic activity of some of the active chains of the complex toxins is higher than that of the relevant intact complex itself. This is particularly true of crotoxin [57] and seems true of taipoxin [88]. There is, then, a clear implication that an inhibitor of PLA_2 is present in some, and maybe in all, complexes. The crotoxin complex has been best studied in this respect. Crotoxin B is much less toxic than the crotoxin complex. For example, the mouse s.c.LD_{50} value of crotoxin is 0.5 mg/kg, whereas that of crotoxin B is 100 mg/kg. And yet, the hydrolytic activity of crotoxin B is approximately an order of magnitude higher than that of crotoxin. Crotapotin, the smaller subunit of the crotoxin complex, is non-toxic and has no hydrolytic activity. The combination of crotoxin B with crotapotin results in the restoration of toxicity but a reduction of the hydrolytic activity of crotoxin B. The acidic PLA_2 of crotoxin (which is non-toxic) is not inhibited by crotapotin.

Effects on transmitter release. Most information on the effects of the basic neurotoxins with PLA_2 activity has been derived from experiments made on vertebrate skeletal nerve-muscle preparations. The toxins seem to share several properties. For example, there is a lag-period of between 5 and 20 min before any effect on transmission is noted. The duration of the lag-period tends to be reduced when preparations are stimulated continuously, as does the rate at which transmission is suppressed [51,89,90]. The lag-period probably represents a time during which the toxins are becoming bound to the nerve-terminal membrane, since removal of excess toxin by washing during this period has little measurable effect on either the rate or subsequent degree of neuromuscular block [90–92]. The rate of block is also temperature- and concentration-dependent [90–92].

There seem to be marked species differences in sensitivity to the toxins [90,93,94], but it is important to note that there have been few systematic investigations of the differential susceptibility of a single species to the full range of toxins or of a range of species to a single toxin. Acute toxicity tests have shown that β-bungarotoxin is less toxic to neonatal rats than to 15-day-old animals [95], but it is not clear whether this is a general phenomenon.

Although most initial screening experiments concerned with presumed neurotoxins involve making measurements of the twitch tension generated in response to indirect (i.e., nerve) and direct (i.e., muscle) stimulation, these are difficult experiments to interpret because they do not readily allow the differentiation between postsynaptic and presynaptic factors involved in the failure of neuromuscular transmission. More accurately, effects on synaptic trans-

mission in nerve-muscle preparations are assessed by measuring the frequency of miniature end-plate potentials (mepps) and the amplitude of evoked end-plate potentials (epps). A statistical analysis of amplitudes of mepps and epps allows the quantal content of epps (m) to be calculated. These techniques are now well known, and rely on the interpretation that the mepp is generated as the consequence of the reaction between the postsynaptic receptors and a 'quantum' of transmitter. The epp is generated as the consequence of the interaction between the receptors and a number of quanta (m) released in a more or less synchronous manner by the nerve impulse.

The vesicle hypothesis suggests that individual quanta of transmitter are contained within the vesicles seen in the presynaptic nerve terminals of typical chemical synapses.

At many synapses, several populations of mepps may be recorded. The most common are those whose respective amplitudes form a normal (i.e., Gaussian) distribution with a coefficient of variation of about 25%. The actual amplitude depends on a number of factors, including the resistance of the postsynaptic membrane as well as the amount of transmitter contained in a quantum, but is typically in the range of 0.2 to 1.0 mV. It is this population of 'classical' mepps to which most authors refer when writing of mepps. Minor populations of mepps (at least in the majority of circumstances) comprise the so-called 'giant mepps', usually considered to be multiquantal in nature, and the so-called sub-miniature mepps. These mepps may be the 'true' quantal unit, or they may represent a population of non-quantal mepps [96,97].

Except where stated otherwise, all references to mepps in this review are presumed to relate to the 'classical' mepps, although it is important to bear in mind that few authors make any particular distinction between the various populations of mepps.

It is commonly claimed that transmission failure following the exposure of nerve-muscle preparations to the presynaptically active neurotoxins is triphasic (Refs. 94, 98, for example), but this is an oversimplification; the precise sequence of events leading to total failure of transmission varies with species, toxin and the circumstances of the experiment.

With respect to spontaneous release, a triphasic response is seen in frog nerve-muscle preparations when exposed to β-bungarotoxin, notexin and taipoxin. The first phase is an initial suppression of release which lasts for between 5 and 20 min. This is followed by a phase of enhanced release lasting for 60–120 min. The final phase consists of a slow but steady reduction in release, resulting in near-total failure [99–101]. The initial phase of suppression is not generally seen in rat, mouse or chick muscles exposed to β-bungarotoxin [5,92,102] although there are reports to the contrary [103,104].

Similarly, the first phase is absent when rat and mouse preparations are exposed to crotoxin; the second phase is also absent in rat preparations [90].

Neither taipoxin nor notexin results in a phase of enhanced transmitter release in rat nerve preparations [89,105], although 'bursts' of high-frequency mepps may be seen in rat muscles exposed to taipoxin [89]. Crotoxin has been reported to initiate 'bursting' in mouse preparations [90] and β-bungarotoxin in frog muscles [99].

When lethal doses of the toxin are administered to experimental animals, and nerve-muscle preparations are isolated *in extremis*, mepp frequency is typically reduced [89,105], but may be unchanged [91]. Mepp frequency in intoxicated preparations is not enhanced by high external concentrations of potassium [91,105].

Little attention has been paid to the effects of the toxins on non-classical mepps, but it has been reported that β-bungarotoxin does not block the release of sub-miniature mepps [103] in mouse nerve-muscle preparations, and it has been implied that taipoxin increases the release of giant mepps in rat nerve-muscle preparations [89]. It is difficult to be certain of this latter point, however, since taipoxin also promotes bursts of mepps. When these bursts are of sufficiently high frequency, summation may occur, giving rise to a response with many characteristics of a giant mepp [99].

Generally, evoked transmitter release follows the same pattern as spontaneous transmitter release, but tends to fail totally *before* the cessation of spontaneous release.

Exposure of frog muscle to β-bungarotoxin may result in the generation of spontaneous end-plate potentials [99]. Such a response does not appear to have been reported elsewhere.

In nerve-muscle preparations exposed to presynaptically active PLA_2 toxins for periods of 30 min, ultrastructural evidence of physical damage is not uncommon. The damage may be very severe, giving rise to acute denervation [99], or less severe, with some limited mitochondrial damage, depletion of vesicles and the presence of Ω-shaped identations in the nerve terminal membrane – often interpreted as indicative of an inhibition of vesicle recycling [106,107].

Neuroeffector transmission in the autonomic nervous system is largely unaffected by the presynaptically active PLA_2 neurotoxins. Cholinergic transmission via the vagus is blocked by β-bungarotoxin in the guinea-pig atrium [108] and in the superior cervical ganglion of the cat [109]. In the latter, transmission block is associated with a reduction in evoked release of transmitter, although spontaneous release is slightly potentiated. Notexin, taipoxin and β-bungarotoxin will block neurogenic contractions of the guinea-pig vas

deferens and seminal vesicle (Refs. 94, 110, and Harris, unpublished data). In the vas deferens preparation, the mechanism is almost certainly presynaptic in origin, and derives from a toxin-induced reduction in the amplitude and facilitation of excitatory junctional potentials (Harris and Surprenant, unpublished data). There are marked species variations in the sensitivity of various autonomic neuroeffector systems. For example, β-bungarotoxin will not block the effects of vagal stimulation in mouse, rat or rabbit [111].

It should be noted that, although it is argued on pharmacological grounds that all the examples of autonomic transmission block cited above are the result of an inhibition of transmitter release, in only one example has it been unequivocally shown that evoked release is blocked [109].

The fact that the presynaptically active toxins block transmitter release at vertebrate neuromuscular junctions and that some at least block the release of transmitter release in the superior cervical ganglion of the cat, which is also a cholinergic synapse, has led to a widespread belief that the toxins block only cholinergic systems. This is not true. None of the toxins has been shown to block neuromuscular transmission in the longitudinal muscle of the guinea-pig ileum [16,109,110], which employs a cholinergic synapse, but most appear to block motor transmission in the guinea-pig vas deferens [94,110], which is certainly non-cholinergic.

The effects of the toxins on transmission in the central nervous system are even less well documented than those in the autonomic nervous system. There are two plausible reasons for this. First, the central nervous system is rather more difficult to explore in a meaningful manner than the autonomic nervous system; second, most toxinologists consider it to be of little practical interest, since in natural circumstances (i.e., following an evenoming bite) few toxins cross the blood-brain barrier.

In isolated slice preparations of rat and guinea-pig olfactory cortex, electrical stimulation of the lateral olfactory tract results in a complex electrical response which may be recorded extracellularly from the cortex. The response comprises the compound action potential of the olfactory tract, followed by a slow negative potential, the N-wave, which represents the electrical activation of the cortical cells. Superimposed upon the N-wave is the positive-going P-wave, which represents the discharge of the excited cortical cells. β-Bungarotoxin appears to block the N-wave but not to change the sensitivity of the cortical cells to putative transmitter glutamate or aspartate [112]. Similar observations were made on transmission in slices of hippocampus [113]. The data are interpreted as indicating that the toxin inhibits transmitter release from the primary afferent systems. Unfortunately, the actions of β-bungarotoxin in the CNS are not very specific and arguments about the precise locus of action are

largely based on the rate at which the various components of the field potential are blocked by relatively high concentrations of toxin (up to 230 nM). The experiments should be taken as suggestive of presynaptic activity in the central nervous system, but the results should be treated with caution.

Although there is no doubt about the presynaptic activity of the PLA_2 neurotoxins, very little is known of their mechanism of action. Investigations on this matter have centred around two quite distinct questions. The first may be stated rather simplistically – is the reduction in transmitter release the result of a disruption of the accumulation, synthesis or storage of transmitter or is structural damage to the motor nerve terminal involved? The second is concerned with the role of PLA_2 activity itself.

Transmitter accumulation, synthesis and storage. The effects of the neurotoxic PLA_2 enzymes on the accumulation of transmitter or transmitter precursors have been most frequently studied on cell-free systems such as synaptosomes, mitochondria and vesicles of skeletal muscle sarcoplasmic reticulum (SR). Experiments on synaptosomes have shown that preincubation with the toxins results in an inhibition of the high-affinity uptake of choline and also the uptake of GABA, noradrenaline, 5-HT and deoxyglucose [114–118]. As a result of such investigations, it has been claimed that the primary cause of transmission failure is a suppression of the reaccumulation of transmitter or transmitter percursor. More recently, however, it has been found that the simultaneous incubation of synaptosomal preparations with choline and either β-bungarotoxin, notexin or notechis II-5 may result in an increase in high-affinity choline uptake [119–121].

There are marked species differences in these effects of the toxins. For example, high-affinity choline uptake into guinea-pig cortical synaptosomes is stimulated by exposure to either notexin or notechis II–5, whereas uptake into *Sepia* synaptosomes is unaffected [119]. Moreover, it is highly probable that the effect of a given uptake process varies with toxin concentration, temperature and duration of incubation; if a clear understanding of the effects of the toxins of synaptosomal uptake processes is to emerge, the effects of these variables need to be carefully assessed.

The inhibition of uptake, once considered highly specific for choline (see Ref. 119, for example), is clearly nonspecific in the sense that a number of transmitter substances are involved. Moreover, the inhibition probably does not result from a direct effect on the carrier processes, since there is unequivocal evidence that in some experimental protocols used the toxins resulted in the physical destruction of the synaptosomal preparations [116]. In other protocols, indirect evidence for a fall in synaptosomal membrane potential – which

would inhibit most high-affinity uptake processes – has been obtained [122,123]. The fall in resting potential does not seem to be Na^+-dependent, and presumably reflects a nonspecific increase in ionic permeability [123].

Relatively few data are available on the effects of the toxins on transmitter synthesis in cell-free systems. Preincubation of rat cortical synaptosomes with β-bungarotoxin results in a reduction in ACh synthesis, and since neither choline acetyltransferase (CAT) nor AChE activity is changed, the reduction in synthesis might be considered to reflect the inhibition of high-affinity choline uptake recorded in the same experiments [117]; the simultaneous incubation of rat-brain synaptosomes with notexin and choline results in an increase in ACh synthesis which might be correlated with the increase in choline uptake observed under these circumstances [121].

The storage of transmitter substances by synaptosomes is impaired by the toxins, and as a result there is an enhanced release of accumulated transmitter [8,115–117]. This release may be partially Ca^{2+}-dependent [116], but since so many experimental protocols cause physical damage to the synaptosomes, it is difficult to know how important this dependence is. It is of interest that the so-called T-sacs of *Torpedo* electric organ do not release transmitter when exposed to either taipoxin or β-bungarotoxin [119] and that notexin may actually inhibit the release of newly synthesized ACh from rat-brain synaptosomes [121].

The use of mitochondria and vesicles of SR as 'model' systems on which to study the effects of the neurotoxic PLA_2 toxins is unlikely to be helpful. The rationale behind making the experiments is related to suggestions that the effects of the toxins on the function of nerve terminals involves an inhibition of mitochondrial respiration and a reduction in the ability to accumulate or retain Ca^{2+} (see Ref. 124, for example). There is little doubt that Ca^{2+} uptake is blocked in rat brain mitochondria, and in SR [124–126], but there is no evidence at all that the toxins can enter intact synaptosomes or nerve terminals. Further, all available (albeit limited) evidence suggests that the toxins are active on the external surface of the plasma membrane [119,127,128].

β-Bungarotoxin has been shown to stimulate the synthesis and accumulation of ACh in nerve-diaphragm preparations. The mechanism by which transmitter synthesis is stimulated is unclear, and all that is so far known is that CAT activity remains unchanged [129,130]. The increase in tissue levels of ACh seen after exposure to β-bungarotoxin is independent of nerve stimulation, and precedes the toxin-induced inhibition of evoked release. The accumulated ACh cannot be released by K^+, by black widow spider venom, or by nerve stimulation in the presence of 4-aminopyridine [130]. Notexin does not have the same effect as β-bungarotoxin or transmitter synthesis and accumulation

in the rat nerve-diaphragm; rather than stimulating synthesis, tissue levels of ACh are depressed by notexin [130].

How the data on transmitter synthesis and storage relate to the physiological data on transmitter release at the nerve-muscle junction is unclear. The initial response of the toxins is to inhibit transmitter release. There is no obvious explanation for this. Even a rapid inhibition of high-affinity choline uptake (by hemicholinium, for example) does not result in an immediate reduction in mepp frequency [131].

The rise in mepp frequency seen as the second phase may be related to the depolarization caused by the toxins and the increase in quantal content of epps could be related to the same phenomenon as a result of a depolarization-linked influx of Ca^{2+} into the nerve terminal [131,132]. In the longer term, transmitter release might cease as a result of a combination of vesicle depletion, cessation of vesicle recycling, and a Ca^{2+}-activated degeneration of the nerve terminal. Although these mechanisms have been discussed at length in numerous articles (see Refs. 99, 104, 106, 128, for example), there is no firm evidence in favour of any of them and no model that will successfully deal with the varied nature of the data available.

It is generally accepted that the PLA_2 activity of the presynaptically active toxins is essential for the expression of neurotoxicity. This view is based on the observation that all of the snake venom toxins that inhibit transmitter output are phospholipases and that no toxin has yet been modified in such a way that neurotoxicity is retained in full, in the total absence of hydrolytic activity.

There is little doubt that PLA_2 activity is not the sole determinant of toxicity, since the majority of snake venom PLA_2 enzymes are relatively non-toxic [77,94], but the precise details of the relationship between neurotoxicity and PLA_2 activity remain incompletely understood, and it is extremely difficult to design an adequate experimental protocol to explore such a relationship. At first sight, it would appear that the most sensible procedure would be simply to remove Ca^{2+} from a bathing or incubating medium, since that should result in the abolition of hydrolytic activity. This cannot easily be done, however, since many biological processes – including those involved in transmitter release – are also Ca^{2+}-dependent. The procedure usually adopted, therefore, has been to use Sr^{2+} in place of Ca^{2+}, because this exchange has no effect on transmitter release, but results in the inhibition of hydrolytic activity. The inhibition of hydrolytic activity is not always total [133–137]. Nevertheless, it is widely held that if hydrolytic activity is suppressed, presynaptic activity is also suppressed. Although this may be true in general terms, the data are open to misinterpretation for two major reasons. First, hydrolytic and biological activities are rarely, if ever, assayed on the same preparation; second, most

assays of hydrolytic activity are made on either phosphatidylcholine or phosphatidylethanolamine, but it is not at all clear that these are relevant lipids [77].

A second approach has been to prepare derivatives of the toxins and to monitor changes in hydrolytic and biological activity. The most commonly used derivative is that prepared by incubating the relevant toxin with *p*-bromophenacyl bromide (PBP). This process was shown to inactivate porcine pancreatic PLA_2 by the modification of a single histidine residue (homology position 48) at the active centre of the enzyme [138]. The modification does not appear to change the tertiary structure of the toxins [88], but the binding of Ca^{2+} is suppressed. As a generalization, it is true that derivatization of the presynaptically active toxins results in the abolition of hydrolytic activity and of biological activity [83,88,139], and although very large concentrations of the modified toxins will express most of the actions of the native toxins, this probably reflects the preservation of low levels of PLA_2 activity [83,88,139].

The suppression of hydrolytic activity by inhibitory divalent cations does not inhibit binding of the toxins to the membrane-bound target site [140], but whether PBP-derivatives bind to the target site is unclear [84,110,140].

Ethoxyformic anhydride (EOFA) can be used to abolish the hydrolytic and biological activities of β-bungarotoxin and notechis II-5 [141,142]. Derivatization with EOFA does not alter Ca^{2+}-binding. Incubation of β-bungarotoxin with EOFA in the presence of a high concentration of Ca^{2+} and a suitable substrate for the PLA_2 activity of the toxin results in the loss of neurotoxicity, but the preservation of hydrolytic activity [123,141]. These experiments clearly indicate that the 'enzymatic' site and the 'neurotoxic centre' of the toxin are distinct. Unfortunately, such a separation of different forms of activity has not been successful in the case of notechis II-5 [142]. Given the large number of potential sites with which EOFA could react (it acetylates histidine residues and free amino groups), it is difficult to establish the biochemical basis of the differences between the two experiments [142].

If it is accepted that PLA_2 activity is essential for the neurotoxicity of the presynaptically active toxins, it becomes possible to explain the biological activity. Thus the binding of the toxins in the presence of Ca^{2+} leads to the hydrolysis of nerve-terminal lipids. If the most susceptible lipids are those responsible for ensuring the stability and function of plasma-membrane-bound enzymes or ionophores (such as the annular lipids), the initial response could be the suppression of the active ion transport, and, possibly, an increased leakiness (see Ref. 143, for example) without obvious evidence of physical damage to the membrane. If such a mechanism blocked Ca^{2+} movement, the initial result could be a reduction in transmitter output. As lipid hydrolysis proceeded, and membrane damage was initiated, an early depolarization would

occur because of the generalized movement of ions along their respective concentration gradients. Eventually, as a direct result of the hydrolysis of the membrane lipids, and the indirect result of the liberation of lysophosphatides and free fatty acids and the activation (by enhanced Ca^{2+} levels) of endogenous PLA_2 and neutral proteinases, a total disruption of the nerve terminal and its organelles (such as mitochondria) might be expected [144–147]. It is not necessary to consider that the nerve-terminal organelles are primary targets of the toxins, or even that the toxins act only after internalization.

Structure-activity relationships

There are two aspects of importance to consider with respect to the relationship between structure and activity. The first concerns the role of the various subunits in the expression of activity of the complex toxins and the second concerns the recognition of the active site(s) of the toxins.

The role of crotapotin (i.e., CA) in the crotoxin complex, of β- and γ-taipoxin in the taipoxin complex, of β-bungarotoxin chain A in the β-bungarotoxin complex, and of Mojave-toxin A in the Mojave-toxin complex, is not yet known. The β-bungarotoxin complex is particularly difficult to study because the two chains are covalently linked.

In general, the basic chains (i.e., crotoxin B, α-taipoxin, β-bungarotoxin A and Mojave-toxin B) are the only subunits with marked biological and hydrolytic activity. In the presence of the acidic subunits (i.e., crotapotin, γ-taipoxin, β-bungarotoxin A and Mojave-toxin A), the toxicity of the relevant basic units is enhanced. For example, the toxicity in crotoxin B is increased by between one and three orders of magnitude (depending upon the nature of the assay) following recombination with crotapotin [57]. Similarly, the toxicity of α-taipoxin is increased by between one and two orders of magnitude following recombination of the ternary complex of α-, β- and γ-subunits [55]. Claims that crotapotin suppresses the hydrolytic activity of crotoxin B [57] may not be strictly accurate [148].

The nature of the potentiation of the toxicity of the basic subunits is unclear. It has been suggested that the non-toxic subunits of taipoxin act as chaperones for the toxic α-subunit, "sharpening the specificity and increasing the stability of the toxic protein" [55] as it approaches its normal target site, and an essentially similar proposition has been made to explain the role of crotapotin [149]. If this is the case, it is difficult to understand why neither notexin nor notechis II-5 requires a chaperone. It is clear that the complex need not necessarily be formed before administration, because crotoxin B and crotapotin can be separately injected intravenously into mice with time intervals of up to 30 min

before there is an appreciable loss of toxicity [57], but it is not known whether, under such circumstances, complex formation occurs either in the circulation or *in situ* at the target site. It has been shown that if the crotoxin subunits are covalently linked, hydrolytic activity is retained, but neurotoxicity is lost [150]. This has been interpreted as supporting the 'chaperone concept', but no proponent of the concept has yet explained how the complex is broken at an appropriate binding site. Perhaps of greater significance is the fact that it is not known whether complex formation is maintained for any appreciable time after the administration of a complex toxin to a living animal. This is an important gap in current knowledge, because it is central to the 'chaperone concept'.

How β-bungarotoxin, with its two covalently linked subunits, fits into this general scheme is, at the moment, unclear. Investigations are hindered by the difficulty of reforming an active complex from the separated chains.

Very little progress has been made towards the identification of the active region of the toxins. There can be no doubt that His-448 (homology position) is important, because this is almost certainly the residue that reacts with PBP (see pp. 77 and 87 of this review). If, however, the suggestion that PBP-toxins bind to the target sites with low affinity is correct, then His-48 cannot be uniquely involved and may not even be directly involved in binding. The transformation of β-bungarotoxin from a neurotoxic PLA_2 to a non-toxic PLA_2 following treatment with EOFA [141] suggests that enzymatic centre and binding site are distinct, but the reacting residues were not identified in that work. An extensive series of experiments utilizing notechis II-5 modified with PBP and EOFA was recently reported [142]. The results were disappointingly inconclusive, but the paper should be read for its objective discussion of the difficulties involved in handling one of the more 'simple' PLA_2 toxins.

TOXINS THAT POTENTIATE TRANSMITTER RELEASE

Introduction

Although several spider venoms are capable of enhancing transmitter release from motor nerve terminals [151], such activity in snake venoms is highly unusual. It was, therefore, of considerable interest when it was found that the venom of the Eastern green mamba, *Dendroaspis augusticeps* was capable of facilitating neuromuscular transmission [152]. A single fraction of the venom, known as dendrotoxin, appears to be responsible for the facilitation [153]. A similar toxin, toxin 1, has been isolated from the venom of the black mamba, *Dendroaspis polylepis* [154–156].

Structure

The toxins are strongly basic single-chain polypeptides of 59 or 60 residues. They are rather rich in arginine residues (containing seven or eight) and are cross-linked by three disulphide bridges [153,154]. Nothing is known of their secondary or tertiary structure.

Pharmacological properties

Only a relatively crude fraction of dendrotoxin has yet been used to obtain detailed information on the action of these toxins [157]. The frequency of spontaneous transmitter release is firstly slightly reduced, then returns to normal before being steadily potentiated. After 1–2 h exposure to the 'crude dendrotoxin', the frequency of mepps is, on average, increased 5-fold. During the period of suppression, the proportion of giant mepps is increased, leading to an increase in the calculated mean amplitude of mepps. This is only a transient phenomenon, the proportion of giants falling again after only 30 min exposure to the toxin.

There is no temporary suppression of evoked transmitter release. The calculated quantal content of the end-plate potential steadily increases to levels as high as 40-times that of control after 2 h of exposure.

The toxins have no effect on the resting potential of the muscle fibres, and since the rise times of the epps were unchanged, it is probable that there are no major changes in membrane capacitance.

There is, to date, no explanation for these changes in transmitter release. It has been suggested that the toxin induces the clumping or fusion of vesicles, and that this would explain both the emergence of giant mepps and the increase in quantal content of evoked epps [157]. This seems unlikely, however, because the increase in the proportion of giant mepps is transient, whereas the increase in quantal content is long-lasting. An increase in the duration of the nerve terminal action potential would result in an increase in quantal content, without necessarily producing any change in mepp frequency, but this would not explain why there is an increase in the proportion of giant mepps. Further work on these interesting toxins may indicate that the effects on spontaneous and evoked transmitter release are mediated via two distinct mechanisms.

It has been suggested that the two toxins act in a synergistic manner, but that they may not share a common mode of action [156,158]. The basis on which these suppositions are made is rather tenuous.

Structure-activity relationships

Nothing is known of the structure-activity relationships of dendrotoxin or toxin 1.

MYOTOXINS

INTRODUCTION

Effective envenoming bites by many snakes of families Crotalidae and Viperidae give rise to local necrosis involving skin, connective tissue and skeletal muscle [6–8]. Effective bites by sea-snakes (and especially by *Enhydrina schistosa*) give rise to very severe myonecrosis [5], and despite the dogma that the venoms of the Elapidae are 'neurotoxic', bites by many elapid snakes also cause myonecrosis giving rise to myoglobinuria, an increase in the release of muscle-specific enzymes, and severe muscle pain. The elapid snakes known to cause such problems in man include *Naja nigricollis*, *Naja naja* (sub-species *kaouthia* and *leucodira*), *Pseudechis australis*, *Notechis scutatus*, *Cryptophis nigrescens*, *Oxyuranus scutellatus* and *Oxyuranus microlepidotus* (see Ref. 2).

The first potent myotoxin to be properly characterized was notexin, a toxin isolated from the venom of the Australian tiger snake *Notechis scutatus* [52]. The demonstration of myotoxicity was accidental. When first isolated, notexin was classified as a presynaptically active neurotoxin on the basis of its effects on the neuromuscular junction [105]. These latter experiments had been made on nerve-muscle preparations isolated from animals given a large dose of toxin by intravenous injection. The myotoxicity was revealed when a low dose of the toxin was administered by subcutaneous injection in the hope of producing a botulinum-like prolonged inhibition of transmitter release [159]. The significance of the choice of route of injection and experimental design is discussed later, but the serendipitous nature of the observation is noted here to emphasize that most, if not all, myotoxic fractions are only relatively specific, and that many toxins will damage soft tissue if administered in large enough quantity.

It is difficult to devise a formal definition of a myotoxin. Ideally, a definition should include a qualification with respect to dose, but this is not practicable, since there is, at the moment, no accepted standard assay for 'myotoxicity'. The choice of toxins for inclusion in this part of the review is therefore arbitrary. All toxins satisfy the following criteria: they cause a local myotoxic reaction in the absence of systemic toxicity when administered by s.c. or i.m. injection, they are isolated from venoms known to cause soft-tissue necrosis in man, and they are active in the rat at a dose of less than 50 μg/kg.

STRUCTURE

To date, seven myotoxins have been isolated and at least partially characterized (*Table 2.3*) but they do not form an homogeneous group. Five of them,

Table 2.3. MYOTOXIC TOXINS

Toxin	*Venom source*		*Phospho-lipase activity*	*Proteo-lytic activity*
	Species	*Family*		
Notexin	*Notechis scutatus*	Elapidae	+	–
Notechis II-5	*Notechis scutatus*	Elapidae	+	–
Taipoxin	*Oxyuranus scutellatus*	Elapidae	+	–
Toxin VI-5	*Enhydrina schistosa*	Hydrophiidae	+	–
Crotoxin	*Crotalus durissus terrificus*	Crotalidae	+	–
Viriditoxin	*Crotalus viridis viridis*	Crotalidae	–	+
Myotoxin$_a$	*Crotalus viridis viridis*	Crotalidae	–	–

notexin, notechis II-5, taipoxin, toxin VI-5 and crotoxin, are structurally closely related and these I shall call Type 1 neurotoxins. They are all basic toxins with PLA_2 activity. Most are presynaptically active, but toxin VI-5, from *Enhydrina schistosa*, though a potent myotoxin, in only mildly neurotoxic [160]. The structural characteristics of all but toxin VI-5 have been discussed in detail in p. 75 of this review; toxin VI-5, a single chain of 120 amino-acid residues, possesses 14 half-cystines and is homologous with the other presynaptically active toxins [161,162].

The remaining two toxins, viriditoxin and myotoxin$_a$, share little in common. Viriditoxin has been isolated from the venom of the prairie rattlesnake *Crotalus viridis viridis*. It is an acidic, mildly proteolytic, polypeptide of 1018 amino-acid residues [163]. Its primary structure has not been determined. Myotoxin$_a$, like viriditoxin, has also been isolated from the venom of the

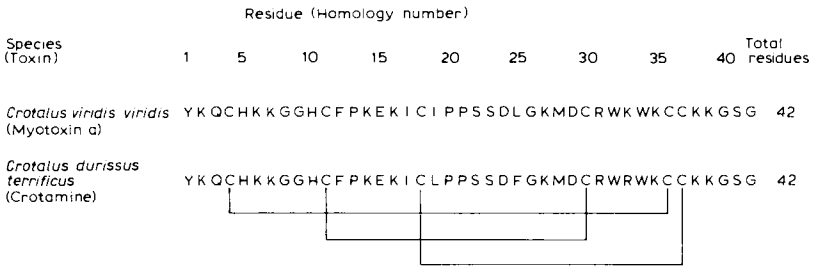

Figure 2.6. Sequences of myotoxin$_a$ and crotamine. The bars link the half-cystine residues involved in the formation of disulphide bridges.

prairy rattlesnake. It is a basic polypeptide of 39 or 42 amino acids organized in a single chain. It is devoid of hydrolytic activity. The reasons for the uncertainty concerning the number of residues in myotoxin a are difficult to understand, because the two estimates were made in the same laboratory [164,165], but the more detailed information available in the latter work [165] suggests strongly that the '42' residue value is probably accurate. It is of interest that in sequence it is very similar to crotamine (see pp. 75–76 of this review), an occasional constituent of the crotoxin complex (*Figure 2.6*).

PHARMACOLOGICAL PROPERTIES

Type I myotoxins

The PLA_2 and the presynaptic activities of the Type I myotoxins have been discussed in detail, and the characteristics of the myotoxicity will now be described.

Following the local administration of the toxins, the first sign of necrosis is the pale and oedematous appearance of the underlying muscle. The oedema is associated with an increase in wet weight which is typically maximal by 12–24 h, and may double the normal weight of muscle. Significantly, the oedema is not accompanied by extravasation (i.e., the loss of intact erythrocytes into the extravascular space). Muscle degeneration is complete by 2–3 days; thereafter, the necrotic muscle fibres regenerate [159,166–169].

At the ultrastructural level, the degeneration of the muscle is associated with hypercontraction of the sarcomeres and the disintegration of the plasma membrane. The basal lamina and the satellite cells of the muscle fibres are undamaged [170–172].

The distribution of damaged and undamaged fibres in muscle exposed to the toxins varies according to the type of muscle under examination. In rat soleus muscles, which consist almost exclusively of Type I fibres (slow-twitch, oxidative) with varying proportions of Type IIa (fast-twitch oxidative-glycolytic) and Type IIc (intermediate) fibres, there may be total destruction of the muscle. If, because of muscle bulk relative to dose of toxin, damage is less than total, a sharp division between a superficial cuff of damaged fibres and a core of undamaged fibres is seen. In the extensor digitorum longus (EDL) muscle, which consists largely of Type IIa and IIb (fast-twitch, glycolytic) fibres, the toxins leave undamaged the IIb fibres, and this results in a 'chequerboard' of damaged and undamaged muscle fibres.

Immature muscle growing in tissue culture and the intrafusal muscle fibres of the muscle spindles are also resistant to the toxins [159,170]. The lack of extravasation further suggests that the microcirculation of the muscle is not destroyed, and preliminary evidence confirms that the capillary bed is largely if not wholly undamaged (Fulthorpe and Harris, unpublished data); muscle damage is therefore unlikely to be due to ischaemia.

The muscle damage does not appear to be causally related to the neurotoxic effects of the toxins. Thus, acutely and chronically denervated muscles are as sensitive to the toxins as are innervated muscles [159]. Moreover, some closely related neurotoxins (such as β-bungarotoxin) are devoid of measurable myotoxicity [170].

The mechanism whereby the Type I myotoxins precipitate muscle damage is unclear. The loss of the plasma membrane is generally thought to result from the hydrolysis or solubilization of the plasma membrane lipids [78]. An alternative view might be that fragmentation of the plasma membrane is related to the hypercontraction of the sarcomeres. This is not entirely unreasonable. It is known that the hypercontraction can be so severe that myofibrils can be torn apart, leaving hypercontracted clots of myofibrils separated by areas of empty basal lamina tube [173]. If the Z-disc material is attached to the plasma membrane through bridges of desmin [174,175], it might be anticipated that the rupture of the myofibrils could result in the fracture of the plasma membrane. Whatever the cause, the loss of integrity of the plasma membrane would be expected to lead to a nonspecific increase in membrane permeability, and a collapse of all ionic gradients existing across the plasma membrane. The resulting influx of Ca^{2+} in particular would activate or further stimulate the contraction of the myofilaments, would poison mitochondria by Ca^{2+} overload [176], and may cause the autolysis of the muscle fibre by activating endogenous PLA_2 and the Ca^{2+}-activated neutral proteinases [177]. A schematic diagram of such a process is given in *Figure 2.7*.

Physical damage to the plasma membrane may not be the primary event. It is possible that the hydrolysis of a specialized lipid (such as the annular lipids that seal proteins into the lipid bilayer) in the plasma membrane leads to the development of a leaky membrane without leaving evidence of structural damage, and that this leakiness results in the activation of the autolytic process. To date, these schemes have to be considered speculative, and attempts to identify the more important steps have been rudimentary (see Ref. 167, for example).

The alternative view to the schemes outlined above is that the toxins are internalized – probably by the process of endocytosis [35] – and then released into the cytosol, where they are free to disrupt the function of the mitochondria

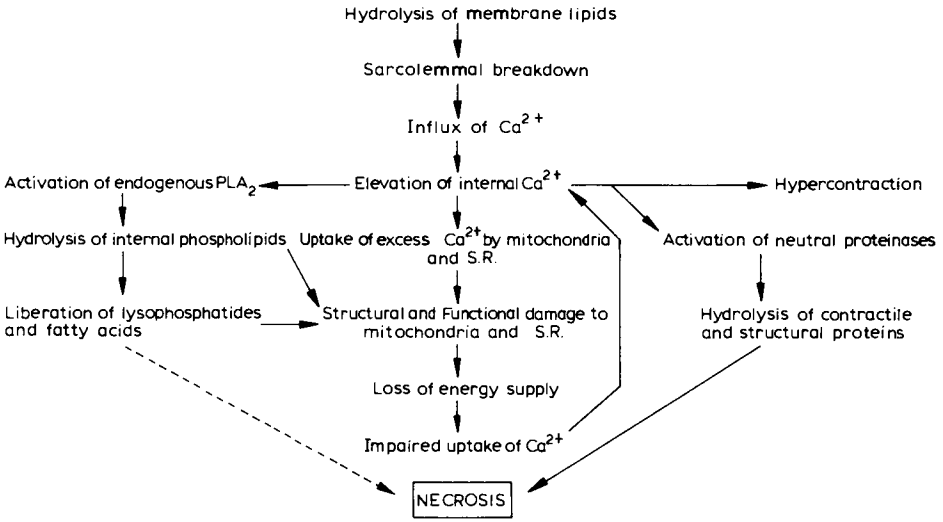

Figure 2.7. A possible mechanism linking the hydrolysis of plasma membrane lipids to muscle degeneration.

and the SR. That the toxins can damage these organelles *in vitro* is well known (see Refs. 125, 126, for example) and one would anticipate that severe damage *in situ* would lead to the loss of Ca^{2+} stored in such organelles with a resulting rise in internal Ca^{2+} levels, and the activation of autolytic processes. There is no evidence that the toxins are internalized.

It is obvious that the alternatives outlined above depend explicitly or implicitly on the assumption that the PLA_2 activity of the Type I myotoxins is essentially involved in the initiation of muscle damage. Evidence in favour of the role of PLA_2 activity is indirect but persuasive. It is reminiscent of that used to relate PLA_2 activity to neurotoxicity (see pp. 79–88 of this review). Thus the removal of Ca^{2+} from an incubating medium results in the loss of myotoxicity *in vitro*, and any chemical modification to the toxin that reduces PLA_2 activity also reduces myotoxicity [78,166]. The weaknesses in the argument are, first, that no quantitative assay has yet been made of lipid hydrolysis versus muscle fibre damage for even a single toxin and, second, that one cannot be sure that the primary target for the hydrolytic activity is one or more of the predominant membrane lipids (i.e., phosphatidylcholine or phosphatidylethanolamine) against which PLA_2 activity is usually measured. It

has been shown that notexin-damaged muscle contains higher than normal levels of lysophosphatidylcholine and lysophosphatidylethanolamine [78], but this does not mean that the hydrolysis of the phospholipids is a primary event.

The pattern of damage caused by myotoxin a is quite unlike that caused by Type I myotoxins. There is neither oedema nor extravasation. The earliest sign of damage is dilatation of the terminal cisterns of the SR [178]. This is followed by the disorganization of the myofilaments and hypercontraction. No detailed information is available on the state of the plasma membrane, basal lamina, Z-disc material or sarcomeres in such muscle fibres, or on the activation of the various phagocytic cells that are normally involved in the clearance of necrotic tissue. Without such information it is impossible to speculate on the mechanism of action of myotoxin a.

Viriditoxin causes muscle damage associated with haemorrhage and extravasation [163], but no details of the pathology are available. The role of proteolytic activity cannot be discussed for lack of relevant information.

STRUCTURE-ACTIVITY RELATIONSHIPS

Without much more information, it is not possible to make any useful comment on structure-activity relationships as they apply to myotoxin a and viriditoxin.

Notexin, notechis II-5 and toxin VI-5 are all single-chain polypeptides. The other Type I myotoxins (taipoxin and crotoxin) are complex toxins of two or three subunits. The only individual subunits which are toxic are those with PLA_2 activity (see pp. 75–76 of this review). These subunits are also the only subunits with myotoxic activity. This has been demonstrated most clearly in the case of taipoxin [168]. Of the three subunits of taipoxin, only the basic α-subunit is myotoxic, neither the acidic γ- nor the neutral β-subunits exhibiting any significant activity at all. The activity of the α-subunit, however, is only 1/10th that of the activity of the ternary complex. Recombination of α- and γ-taipoxin results in the full expression of myotoxicity. The combination of α- and β- results in a partial restoration of myotoxicity, but the combination of β- and γ- is inactive. Similarly, unpublished observations made in the author's laboratory have shown that only crotoxin B of the crotoxin complex is myotoxic, and that the recombination of crotoxin B with crotapotin results in a potentiation of the activity of crotoxin B. Neither γ-taipoxin nor crotapotin will potentiate the activity of the single-chain toxin, notexin.

Few experiments have been made to determine the effects of the chemical modification of the myotoxic toxins on myotoxicity. PBP-notexin is virtually inactive; it does retain some activity, however [166], and it is difficult to

ascertain whether this is due to residual PLA_2 activity or contamination by a trace of unmodified notexin, or whether it represents myotoxicity unrelated to PLA_2 activity. These problems will not be resolved until the precise relationships between PLA_2 and myotoxic activities are known.

It is not understood why certain muscle fibres – such as Type IIb fibres in mature muscles, or immature muscle fibres – are relatively resistant to the toxins. It seems probable that these cells lack either an essential binding protein, or an appropriate membrane substrate for the PLA_2. If it is the substrate that is lacking, it seems unlikely that it is either phosphatidylcholine or phosphatidylethanolamine, because they are major phospholipids in all excitable membranes. To date, nothing is known of the binding sites for these toxins. Further information on the respective identity of the binding site and putative PLA_2 substrates in both muscle and nerve would be enormously helpful to those working on these presynaptically active, myotoxic phospholipases.

CYTOTOXINS

INTRODUCTION

The term cytotoxin is used here as a general term to identify the many toxins variously described as cardiotoxins, cobramines, cytotoxins and direct lytic factors. Elsewhere, toxins of this group are most commonly called cardiotoxins. The latter term appears to have been first used to define a semi-pure fraction of *Naja naja* venom that was found capable of blocking the spontaneous beating of the amphibian isolated heart [179]. It is unsatisfactory because many so-called cardiotoxins do not appear to have any particular affinity for cardiac muscle.

To date, more than 40 toxins of this group have been isolated and sequenced (see Refs. 180, 181, for example). As a generalization, they may be considered typical constituents of the venoms of the Elapidae, and they seem to be particularly important constituents of venoms of the true cobras (genus *Naja*). No toxin of this group has yet been isolated from venoms of the Australian Elapidae.

STRUCTURE

The cytotoxins form a remarkable homologous series. They all comprise 60 or 61 amino-acid residues organized in a single chain cross-linked by four

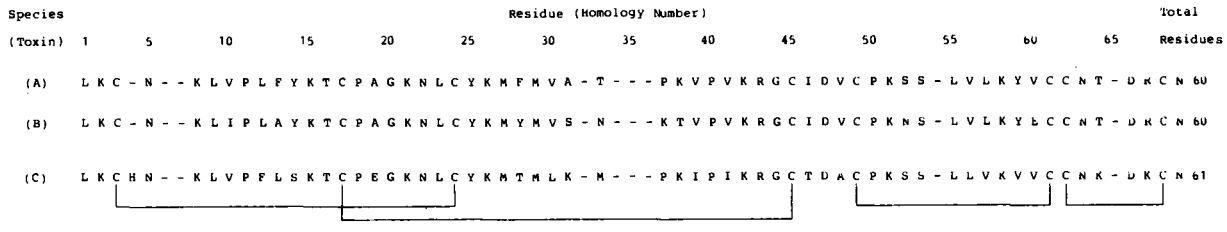

Figure 2.8. Sequences of selected examples of a cardiotoxin, a cytotoxin and a lystic factor. (A) Naja naja atra *(cardiotoxin); (B)* Naja naja *(cytotoxin II); (C)* Haemachatus haemachates *(direct lytic factor). The bars link the half-cystine residues involved in the formation of disulphide bridges.*

disulphide bridges. They are strongly basic compounds, relatively rich in lysine and tryosine residues and relatively poor in arginine and glutamic acid residues. The sequences of three toxins of this group (normally identified respectively as a cardiotoxin, a cytoxin and a direct lytic factor) are illustrated in *Figure 2.8*.

Structurally the cytotoxins are homologous with the postsynaptically active neurotoxins. In particular, the half-cystines occur in the same homology positions and cross-bridging is identical (compare *Figures 2.1* and *2.8*). As a result, it is probable that the secondary and tertiary organization of the cytotoxins is similar to that of the postsynaptically active neurotoxins [182–184].

A series of long-chain toxins (117 or 118 residues) isolated from the crude venom of the banded krait *Bungarus fasciatus* were claimed to represent a new series of cardiotoxins [185–187]. They were classified as cardiotoxins because they caused a contracture of skeletal muscle, and because they appeared to be devoid of hydrolytic activity [188]. It is now known that these toxins have measurable PLA_2 activity and that they are homologous with classical venom PLA_2 enzymes [187,189]. As a result, they should not be considered cytotoxins and they should not be confused with the cytotoxin (known as a 'cardiotoxin-like protein') recently isolated from the venom of the Formosan krait, *Bungarus multicinctus*, which appears to be a true cytotoxin possessing 60 amino-acid residues, including eight half-cystines, and deviating very slightly in composition from a typical 'cardiotoxin' [190].

PHARMACOLOGICAL PROPERTIES

Although the cytotoxic toxins are closely related to the postsynaptically active toxins, they are relatively non-toxic in terms of LD_{50} values. A typical intraperitoneal LD_{50} value of a postsynaptically active neurotoxin is 0.1 μg/g mouse, which is 20–50-times less than the typical LD_{50} value of a cytotoxin. Moreover, the cytotoxins do not possess any marked ability to block neuromuscular transmission by binding to postsynaptic cholinergic receptors.

They do, however, possess a bewildering range of pharmacological properties, including the ability to depolarize and inhibit the contractility of cardiac, smooth and skeletal muscle, to induce irreversible contractures in the same muscles, and to cause the lysis of a variety of cell types such as erythrocytes, Yoshida-sarcoma and Ehrlich ascites tumour cells [188,190–195]. The s.c. or i.m. injection of the cytotoxins may also give rise to a severe necrotizing myopathy [196].

Whether all cytotoxins exhibit all of these various forms of activity is uncertain. A particularly important problem is caused by the difficulties

involved in isolating the toxins in absolutely pure condition. It has often been shown that the cytotoxic toxins stimulate the activity of venom PLA_2 [197, 198], and the recent findings that the strongly basic cytotoxins are frequently contaminated with venom fractions possessing PLA_2 activity [199,200] suggest that in many cases PLA_2 contaminants may be responsible for actions ascribed to cytotoxins. For example, toxin F_8, a so-called cardiotoxin isolated from the venom of *Naja naja siamensis*, is said to cause contractures of muscle and to lyse erythrocytes. If, however, the toxin is subjected to stringent purification (using a combination of ion-exchange, immunoaffinity and hydrophobic chromatography), a PLA_2 contaminant is removed. Purified toxin F_8 is not haemolytic, but it does cause muscle contractures [200]. Not all cytotoxins are haemolytic simply because they are contaminated with PLA_2; toxin 3.20 from the venom of *Naja melanoleuca* and toxin $V^{II}4$ from *Naja mossambica mossambica*, for example, retain haemolytic activity even after very rigorous purification [200].

The direct lytic properties of the cytotoxins have been discussed in detail on a number of occasions [201,202] and are beyond the scope of this review. They will not be discussed further.

There is some dispute on the effects of the cytotoxins on peripheral nerve. It has been claimed that the toxins block axonal conduction and that this activity is potentiated by PLA_2 [193], and it has been claimed that the cytotoxins are rather inactive on peripheral nerve, and serve only to potentiate the effects of venom PLA_2 activity [203,204]. The experiments of the various protagonists are difficult to interpret. First, it is difficult to be sure that the cytotoxic fractions were absolutely pure and devoid of contamination by traces of PLA_2; second, the conditions of the experiments can be inadvertently chosen to produce a given result. Thus, low Ca^{2+} concentrations (i.e., less than 2.0 mM) in bathing media may reduce the specific activity of the PLA_2 and allow the expression of the lytic effects of the cytotoxins, while high Ca^{2+} concentrations (over 10 mM) tend to inhibit cytotoxic activity and potentiate PLA_2 activity. A detailed study of the effects of highly purified cytotoxins on axonal structure and function is clearly needed.

The depolarization of muscle tissue by the cytotoxins is well established. The rate at which the depolarization proceeds is dose-dependent, and may reach such a level (above 20 mV) that the survival of the tissue must be considered doubtful. The depolarization may be prevented by incubating the tissue with high levels of Ca^{2+} [193–195]. The depolarization does not appear to be sodium-dependent, because it is not blocked by tetrodotoxin or by a reduction in external Na^+ [205].

The contracture of muscle tissue is an invariable response to exposure to the

cytotoxins. The response is so common that some toxic fractions have been classified as 'cardiotoxins' on the basis of their ability to cause contractures in skeletal and cardiac muscle [206].

The relationships between toxin concentration, tension development, rate of change of tension and latent period (i.e., the period between toxin administration and the initiation of the contraction) are rather unpredictable, but in general, as the concentration of toxin is increased, the latent period of the contracture shortens, the final tension is increased and the rate at which tension develops is increased [188,190,195]. In some preparations, the muscles relax spontaneously, but contractility is not restored following this relaxation. Neither is contractility restored when the cytotoxins are removed by washing.

The contractures are not prevented by the presence of a wide range of pharmacological agents, including neuromuscular blocking agents, the muscarinic receptor antagonist atropine, anticholinesterases, tetrodotoxin and dantrolene, and may even be generated in K^+-depolarized muscles and in muscles in which the transverse tubules are disrupted [188,195]. The only pharmacological agent able to influence contractures appears to be the proteinase inhibitor, pepstatin [195].

Although there may be either a slight reduction or potentiation in the evoked twitch response of muscle tissue before the onset of contracture, it seems clear that usually the twitch response, whether evoked by direct or indirect stimulation, fails as the contracture develops [188,195].

The contractures appear to be associated with an increase in Ca^{2+} uptake, but the uptake may not be the result of the movement of Ca^{2+} through specific Ca^{2+} channels, since the contractures cannot be blocked by verapamil, diphenylhydantoin or lanthanum [188,195]. These data should be treated with some caution, however, because the so-called Ca^{2+} antagonists are rather poorly understood in terms of their specificity of action and there is no certainty that those used will block the movement of Ca^{2+} through any given population of Ca^{2+} channels in skeletal muscle.

The experimental data make it clear that although depolarization occurs in cytotoxin-damaged muscle, the depolarization is nonspecific. Furthermore, the contracture does not appear to be depolarization-dependent. All of these features can be explained if it is assumed that the cytotoxins produce a rather nonspecific increase in membrane permeability. The consequent movement of ions along their respective concentration gradients would lead to an increase in the uptake of Ca^{2+} into the muscle. The irreversible contraction of the tissue would then be explicable because the increase in internal Ca^{2+} concentration would be expected to initiate a hypercontraction of the myofibrils.

The increase in Ca^{2+} uptake may also stimulate the autolysis of the muscle

(*Figure 2.7*), thus explaining the several reports that the cytotoxins can cause muscle degeneration [195,196,207].

The precise mechanism of action of the cytotoxic toxins in unknown. It has been suggested that the toxins bind to membrane-bound proteins, since under the influence of the toxins the activity of several membrane-bound enzymes such as acetylcholinesterase and $(Na^+ + K^+)$-ATPase is suppressed [208,209]. Such suggestions take no account of the fact that the enzymes are not located in the same 'membrane system'. Acetylcholinesterase, for example, is located in the basal lamina and not in the plasma membrane. Moreover, many membrane-bound enzymes are actually stimulated [201]. Much more detail is required, therefore, before it could be accepted that the toxins bind to a specific membrane protein. It seems more likely that they interact directly with the membrane lipids [210,211]. If this is the case, it could be that the interaction between toxin and membrane lipid leads to the activation of endogenous PLA_2 [212] and the eventual disruption of membrane integrity. Such a disruption would be fully consistent with the observed changes in function of membrane-bound enzymes and with the development of nonspecific changes in membrane permeability [143].

There is, to date, no consensus on the nature of binding between the cytotoxins and the lipid membranes or on the relationship between binding sites and active sites. Such studies will be of importance, however, because, as stated above, it seems probable that the cytotoxins present in the cobra venoms are responsible for the soft-tissue necrosis seen in many victims of cobra bite.

STRUCTURE-ACTIVITY RELATIONSHIPS

It has been pointed out that the most variable region of the cytotoxin molecule is that between residues 29 and 32. When this region is strongly positive, the molecules tend to be cytolytic and when neutral, the molecules tend not to be cytolytic. Since this region is on the long central loop of the molecule, this loop would appear to be of considerable functional significance [200]. How relevant this region of the long central loop is to the effects of the toxins on muscle tissue is unclear, however, since the non-lytic toxin cardiotoxin III from *Naja naja siamensis* venom, which has a net negative change on region 29–32, is a powerful inducer of muscle contractures.

The more toxic cytotoxins exhibit conserved residues at homology positions 27, 29 and 38. The two methionines at positions 27 and 29 (see *Figure 2.8*) appear to be absolute requirements for toxicity and for the ability to initiate muscle contractures [200]. These residues are also on the long central loop of the toxins. One might infer from these observations that the long central loop

is important for the biological behaviour of the toxins, but that there is no evidence it is involved in binding.

In a rather sophisticated series of experiments which explored (a) the accessibility of identified residues of lipid-bound cytotoxins and (b) the binding to lipids of short (5–8 residue) peptides derived from cytotoxins by trypsin digestion, it was found that residues 6–11 or 6–12 were intimately involved in binding [213]. The proposed 'model' of toxin binding based on these observations left the long central loop 'floating' at the interface of the binding site.

It seems probable that the binding site and functional sites of these molecules differ significantly, and that binding may induce a reorientation of the molecule [214]. Further work is needed, however, before structure-activity relationships can be profitably explored.

CONCLUDING COMMENTS

The system of classification used in this review is basically very simple, and yet even with such a simple system, many toxins from snake venoms are difficult to classify. Several factors contribute to the difficulty. The most common of these factors are as follows:

(1) incorrect identification of the snake from which the crude venom was obtained;
(2) contaminated venom samples;
(3) impure toxic fractions;
(4) variation in experimental technique used by different laboratories.

These factors may be briefly considered in turn.

It should be obvious that the venom sample should be obtained from an accurately identified animal. Unfortunately, unless a commercial supplier of venoms maintains his own serpentarium, it is impossible for him to verify the source of his venom. Frequently, he will be dependent upon the activity of local snake catchers whose knowledge of local fauna may be less than good. Moreover, it is not commonly appreciated that the composition of venom within a given species may vary greatly depending on the precise geographical location inhabited by the individual snake. The most striking example of this phenomenon is the coexistence of two populations of the South American rattlesnake, *Crotalus durissus terrificus* with overlapping territories around São Paulo, Brazil. One population elaborates crotamine as a venom constituent, the other does not [62,63]. Similar geographical variations in venom composition are probably present in venom samples from specimens of the Mojave

rattlesnake, *Crotalus scutulatus scutulatus* [215]. On clinical evidence, the problem is certainly more widespread [1].

The second problem is difficult to discuss objectively, but anecdotal evidence is that venoms of rare snakes or of snakes that yield small samples of venoms may be topped up with more common venoms of unidentified snakes. This is not a problem with venoms obtained from most serpentarium owners. If, however, there is any cause to doubt the purity of a venom sample, the supplier should be asked to provide information on the geographic origin of the snake, to state whether the venom was obtained in the field or from a captive animal, and to state whether a given batch of venom came from a single specimen or from a number of specimens.

The third problem is the most difficult to control. The use of sophisticated isolation procedures has made it possible to obtain very pure venom fractions, but it is expensive technology and requires considerable specialist skill. It is advisable not to accept a toxin sample unless it has been isolated by somebody with an established reputation for the isolation of natural toxins. The dangers of using contaminated toxins have been identified in a number of recent publications [77,199,200].

The fourth problem concerns the way in which experiments are made in different laboratories. It is often a minor problem, but it has, at times, been of major significance. For many years, for example, it has been recognized that the most characteristic features of envenomation following bites by sea-snakes are stiffness, muscle pain on movement and muscle necrosis [5]. In view of this consensus, it is disconcerting to note that the majority of laboratory reports on the pharmacology of snake venoms have emphasized peripheral neurotoxicity (see Ref. 2 for references).

It is now clear that the venoms contain both myotoxic toxins and postsynaptically active neurotoxins. All species appear to be sensitive to the latter, but sensitivity to the former varies according to species. Thus part of the discrepancy is the result of species variability. There is, though, another reason: pharmacologists tend to test venoms and toxins by injecting lethal doses into the circulation of experimental animals. This technique allows the expression of neurotoxicity before soft-tissue necrosis – not usually visible to the naked eye for several hours – is obvious.

Similarly, the notexin-like toxins were originally classified as being neurotoxic as the result of the injection of large doses intravenously into rodents. The subcutaneous injection of the toxins in sub-lethal doses reveals the local tissue damage that these toxins can cause.

In conclusion, it might be asked whether the investigation of the toxic fractions of animal venoms has any relevance to the clinician. I would argue

that such investigations are important, for they allow one to interpret biological activity at the cellular, and sometimes at the molecular, level. As a result, they may allow fragmented and confusing clinical information to be analyzed in a much broader context.

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3 Lonidamine and Related Compounds

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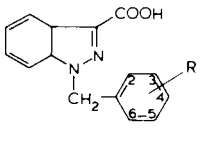
INTRODUCTION

Indazoles may be considered closed models of phenylpyrazoles but, whereas there is a vast literature on arylpyrazole drugs belonging to a variety of therapeutic classes, relatively few biologically active indazoles have been described. This is quite surprising, considering that indazoles are easily synthesized, are of low toxicity and, when properly substituted, exhibit a variety

of pharmacological activities. The first biologically active indazoles were the isosteres of indole-3-acetic acids, which have been shown to be of practical use as growth regulators in plants [1]. As far as medical use is concerned, mention should be made of drugs with characteristic pharmacological properties: for example, benzydamine, an anti-inflammatory drug [2,3]; bendazac, an antinecrotic compound in dermatology [4,5]; the lysine salt of bendazac, an anticataract agent [6–8]; stanozol, an anabolizing agent [9,10]; FS 32, an antidepressant [11]. Some substituted benzothiopyranoindazoles and pyrazoloquinolines [12] with antischistosomic properties have also been described, although they have not yet reached the clinical stage.

The 1*H*-indazole-3-carboxylic acids are a relatively new class of compounds, originally synthesized as part of a chemical project on the indazole ring system structures [13]. *Figure 3.1* shows some of the compounds. 1-*p*-Chlorobenzyl-1*H*-indazole-3-carboxylic acid (AF 1312/TS, 1, R^1 = R^3 = H, R^2 = 4-Cl) was the first compound synthesized and submitted for routine pharmacological screening. The earliest results were disappointing, since they gave no indication of any significant pharmacological action. This screening comprised autopsy on rats following single administration of the chemicals in order to detect late toxic effects. This led to the unexpected observation of a shrinking of the testis. Histological examination of the seminiferous tubules showed marked exfoliation, whereas the spermatogonia and the Leydig cells were found to be normal [14–17]. The specificity of this effect is so remarkable as to make these compounds potentially interesting as male contraceptives. Subsequent studies, however, also revealed other actions of these compounds, including antitumour activity, which in certain aspects appeared even more interesting than the antispermatogenic activity [18].

The aim of this review is to provide a critical overview of the literature available so far on this class of compounds and also of the perspectives of research now in progress.

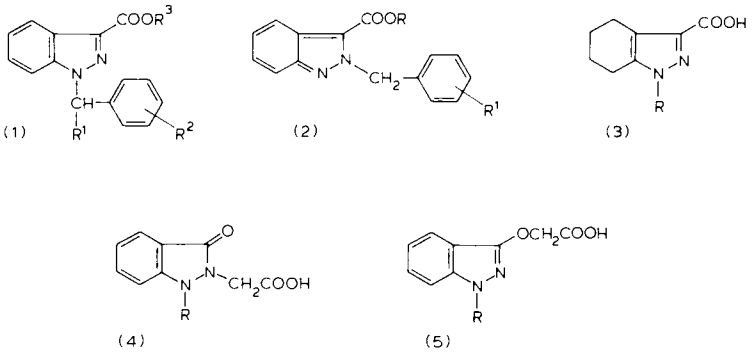


Code number	R
AF 1311	H
AF 1312	4-Cl
AF 1428	4-F
AF 1921	2,4,5-Cl_3
AF 1416	2-Cl
AF 2121	2-CH_3
AF 1915	2,4-$(CH_3)_2$
AF 1914	2,4-Br_2
AF 1890 (Lonidamine)	2,4-Cl_2
AF 1923 (Tolnidamine)	2-CH_3; 4-Cl

Figure 3.1 Structures of some 1-benzylindazole-3-carboxylic acids.

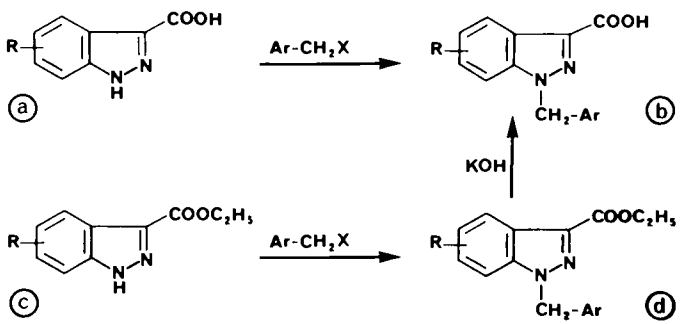
CHEMISTRY

Following the observation that compound AF 1312/TS (1, R^1 = R^3 = H, R^2 = 4-Cl) has antispermatogenic properties, several other molecules were synthesized, including new 1*H*-indazole-3-carboxylic acids (1) [13], 2-benzyl-2*H*-indazole-3-carboxylic acids (2), 4,5,6,7-tetrahydroindazole-3-carboxylic acids (3), indazole-2-acetic acids (4), indazole-3-oxyacetic acids (5) and



1*H*-indazole-3-carboxylic acids in which halophenyl rings are joined to N-1 by means of CO or SO_2 groups. Several indole bioisosteres of these were synthesized.

The new 1*H*-indazole-3-carboxylic acids were prepared following one of the two methods already described [13,19]. The first one consisted in the direct

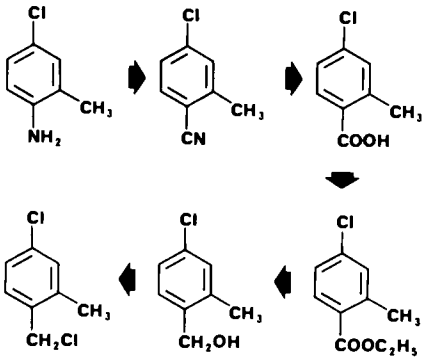


Scheme 3.1.

alkylation of the corresponding 1*H*-indazole-3-carboxylic acids with a benzyl halide in aqueous sodium hydroxide. When this method failed, a second one was used and consisted of the benzylation of the ethyl ester in an ethanol or dioxan solution followed by hydrolysis of the resulting ester with aqueous ethanolic potassium hydroxide (*Scheme 3.1*).

The 2-substituted benzyl-2*H*-indazole-3-carboxylic acids were prepared by benzylation of the ester (c) and subsequent hydrolysis following the methods described by von Auwers and Schaich [20] for the homologous methyl 1*H*-indazole-3-carboxylate. However, while the reaction in ethanol in the presence of sodium ethoxide gave very low yields of 2-benzyl-2*H*-indazole-3-carboxylate and very good yields of the isomeric 1-benzyl product (b), direct heating of ester (c) with the appropriate benzyl chloride without solvent at 120° C gave about equal quantities of the two isomers [21]. The isomers were easily separated by column chromatography or by simple crystallization.

We shall outline the preparation of the two compounds, lonidamine and tolnidamine; both were synthesized from isatin, which was converted into 1*H*-indazole-3-carboxylic acid according to the method described by Snyder, Thompson and Hinman [21a]. 2,4-Dichlorobenzyl chloride was prepared by chlorination of 2,4-dichlorotoluene. The synthesis of 4-chloro-2-methylbenzyl chloride required more steps and is shown in *Scheme 3.2*.



Scheme 3.2.

Lonidamine was prepared by dissolving 1*H*-indazole-3-carboxylic acid (a) in aqueous sodium hydroxide and addition of 2,4-dichlorobenzyl chloride. Acidification yielded lonidamine in 45% yield. Tolnidamine was best prepared from ethyl-1*H*-indazole-3-carboxylate (c). This was treated with sodium hydride and 4-chloro-2-methylbenzyl chloride to give a 50% yield of tolnidamine.

The 1-aroyl-1*H*-indazole-3-carboxylic acids were obtained by treating the 1*H*-indazole-3-carboxylic acid with an aroyl chloride in pyridine solution. The corresponding 1-aroylsulphonyl-1*H*-indazole-3-carboxylic acids were prepared instead from ethyl-1*H*-indazole-3-carboxylate by heating with methyl arylsulphonate in pyridine and in the presence of lithium iodide. 1-Benzyl-1*H*-indazole-3-carboxaldehydes were obtained from the corresponding alcohols by treatment with thionyl chloride and subsequent heating with hexamethylenetetramine. Finally, the derivatives containing α-substituted benzyl groups were synthesized by reaction of ethyl-1*H*-indazole-3-carboxylate with the appropriate α-substituted phenethyl chloride followed by hydrolysis.

These studies have shown that in compounds having the general formula (1) where R^1 = H, the presence of a benzylic group in position 1 is essential for antispermatogenic activity. The presence of one or more R^2 groups as halogens or methyls in the benzyl ring is also necessary. If halogen is the only substituent, its position has little influence on activity; the *ortho* position, on the other hand, is essential in the case of a single methyl group. Highest activity is observed with the two substituents in the *ortho* and *para* positions, regardless of whether both are halogens or one is halogen and the other is methyl. In addition to the acids, other compounds where R^3 is a residue of ethylene glycol or glycerol are active; several other esters were found to be ineffective. Mild activity was also found in unsubstituted amines and hydrazides, whereas the substitution of the carboxylic group with a tetrazole ring or a sulphonic group results in a loss of activity. An alcohol group in place of carboxyl gives an inactive compound, while aldehyde has a similar effect to the acid; neither oxime nor thiosemicarbazone was active. The analogue (1, R^1 = Me, R^2 = 2,4-Cl_2, R^3 = H) of lonidamine was also very active, while a higher homologue (1, R^1 = CH_2CHMe_2, R^2 = 2,4-Cl_2, R^3 = H) was not. Members of the other series were inactive with the exception of a few derivatives having the general formula (5) and the corresponding indazole-3-acetic acids, which have shown moderate activity when substituents at N-1 were the same as those which were effective in compounds of formula (1).

Some indazolecarboxylic acids have been shown to possess antitumour [22–25] and embryotoxic [26] effects. Although these effects have not been systematically studied in the entire series owing to the complexity of the tests involved, information to date suggests that the antispermatogenic, antitumour and embryotoxic actions are closely correlated, as will be shown in the next section. It is, therefore, reasonable to assume that the above-mentioned structure-activity relationships also apply to the antitumour and embryotoxic effects of indazolecarboxylic acids.

PHARMACOLOGY

GENERAL ASPECTS

As usually happens when new compounds are made available by chemical synthesis, the indazolecarboxylic acids were submitted to a broad pharmacological screening; details may be found in the literature regarding representative compounds of this series [13,17,24,27]. Initially, the results of this screening were disappointing. Indazolecarboxylic acids lack the most common pharmacological properties and only at high doses do they produce anti-inflammatory and behavioural effects, consisting of sedation and ataxia. Some of them have a diuretic action, but here again the level of activity is relatively low. For instance, the diuretic doses of lonidamine (*Figure 3.1*) are 40-times higher than those of chlorothiazide. These compounds also lack antimicrobial effects, except on *Mycobacterium tuberculosis*; the MIC (minimal inhibiting concentration) of lonidamine is, however, about 20-times higher than that of rifampicin.

As mentioned previously, the antispermatogenic activity was the first important pharmacological property detected in this chemical class. The first compound found to be active was 1-*p*-chlorobenzyl-1*H*-indazole-3-carboxylic acid (AF 1312/TS) [14–17]. The observation that small chemical changes of this molecule considerably increased the antispermatogenic activity without affecting to the same extent general toxicity or other pharmacological properties [13,24,28] had important consequences on ensuing research. This fact suggested that the mechanism of the antispermatogenic action was highly specific or, in other words, it indicated that the target of these compounds was a biological mechanism playing a critical role in spermatogenesis, but not in other physiological processes; otherwise, derivatives with the most potent antispermatogenic activity would have presented an increase in general toxicity or in other pharmacological properties as well. Consequently, priority was given to the study of the mechanism of action of these compounds.

With this objective in mind, further studies were performed in order to ascertain whether there were any biological or biochemical effects showing a correlation with the antispermatogenic action. This proved to be a success, since it was discovered that indazolecarboxylic acids with an antispermatogenic action also possess embryotoxic [26] and antitumoural effects [22–25]. It was therefore proposed that these three actions have a common mechanism [24]. These properties may be considered the characteristic ones of this class of compounds and will be described in detail. Moreover, biochemical studies showed a good correlation between the antispermatogenic activity of these

compounds and their capacity to inhibit cell respiration [29,30]. These studies were further pursued and the results to date support the working hypothesis that the characteristic properties of these compounds depend on a common and specific mechanism of action [24,31]. This will be commented on in detail later.

CHARACTERISTIC PROPERTIES

Indazolecarboxylic acids are characterized by antispermatogenic, antitumour and embryotoxic properties. The antitumour activity will be described first, since its study is the most advanced; particular reference will be made to lonidamine, which, according to the available information, is the most potent derivative of this series.

In the battery of tests currently used for the screening of antitumour agents, lonidamine has been shown to have a very narrow spectrum of effects [23–25,32,33].

Table 3.1 summarizes its effects on the growth of some well-known cell lines, KB human epidermoid carcinoma cells, P388 lymphocytic leukaemia cells, and HeLa human carcinoma cells.

At the concentration of 10 μg/ml, generally considered to be the threshold of antitumour activity, only growth of the HeLa cells is inhibited [25], whereas their survival is unaffected [32,33].

In the standard transplantable tumours, lonidamine is active only on Sarcoma 180 and Lewis lung carcinoma [23–25], but it is inactive on P388, which, along with some other tumours, has been proposed for a first-stage screening of chemicals [34]. This explains why the antitumoural activity of these compounds was overlooked in their first pharmacological screening.

The above-mentioned tests for the screening of antitumour activity have been set up for anticancer agents which primarily affect the cell division

Table 3.1 EFFECTS OF LONIDAMINE ON THE GROWTH OF SOME CELL SYSTEMS *IN VITRO*

Lonidamine concentration (μg/ml)	*Percent reduction of growth*		
	KB	*P388*	*HeLa*
10	0	0	56
100	74	76	–

process. Lonidamine, however, is not active on rapidly proliferating tissues. For instance, it does not produce bone marrow depression, hair loss, thymus and spleen atrophy or gastrointestinal lesions [27,35]. It is also devoid of mutagenicity, frequently found in compounds which primarily interfere with the genetic make-up of the cell [36]. Available data rather suggest that lonidamine and other indazolecarboxylic acids act on the cell energy metabolism. This raises the question – are the standard screening tests appropriate for the study of this class of compounds or should others be used?

This problem has not yet been resolved, but there is some evidence that if different tests are used, lonidamine shows a more constant antitumour activity. It should be noted that these tests are different from the standard ones, but still mimic conditions actually occurring in the natural cancer pathology. In this connection, the following results may be cited. When Ehrlich ascites cells are treated with low doses of lonidamine before the inoculum, no 'take'

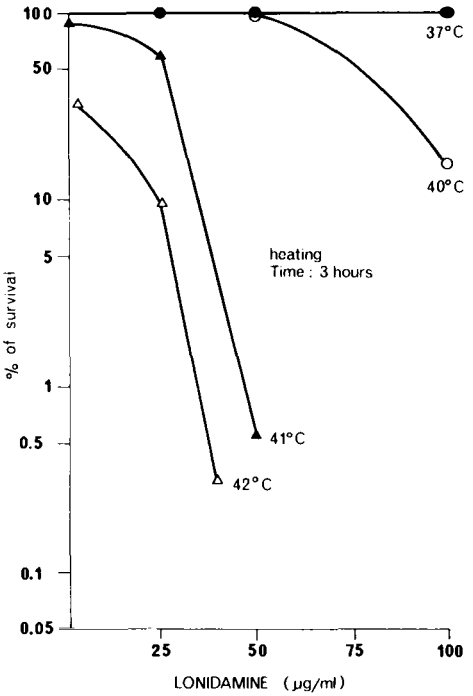


Figure 3.2. An in vitro *study of the effects of lonidamine on HeLa cell survival time at different temperatures.*

occurs [22]. Since lonidamine is inactive on this tumour when animals are treated after the inoculum, this result might indicate that the 'take' is particularly sensitive to lonidamine, at least in the case of transplantable tumours. It has also been demonstrated that the antitumour activity of lonidamine is considerably enhanced under the effect of different agents, such as hyperthermia, low pH and X-rays [25,32,33,37–40]. Hyperthermia was studied both *in vitro* and *in vivo*. The results of a typical experiment *in vitro* are illustrated in *Figure 3.2* [32].

As mentioned previously, lonidamine alone does not affect the survival of

Table 3.2. EFFECTS OF LONIDAMINE AND HYPERTHERMIA (39° C) ON SARCOMA 180 AND EHRLICH ASCITES IN MICE

Tumour	*Treatment*	*Daily dose of lonidamine (mg/kg p.o.)*	*Median survival time (days)*	*ILS*[a] *(%)*
Sarcoma 180	control	0	24.3	
	lonidamine	50	24.1	
	hyperthermia		26.3	9
	hyperthermia + lonidamine	50	31.1	29
Ehrlich ascites	control		18.3	
	lonidamine	25	19.9	9
	hyperthermia		20.4	11
	hyperthermia + lonidamine	25	23.2	27
	control		20.2	
	lonidamine	50	22.2	10
	hyperthermia		23.2	15
	hyperthermia + lonidamine	50	27.4	35

[a] Increase in life-span.

this cell line. When combined with hyperthermia at 41° C (which by itself does not change the survival rate), lonidamine reduces cell survival. Similar results have been obtained *in vivo*; the results of a typical experiment are reported in *Table 3.2* [25].

By combining inactive doses of lonidamine with mild hyperthermia, a marked antitumoural effect is produced. This result confirms those found in *in vitro* tests. Its practical interest lies in the fact that the antitumoural effect of lonidamine is produced at a body temperature of 39° C, easily attainable in humans.

It is interesting to note that in the conditions of hyperthermia used for some of the above experiments, the tumour cells present characteristic ultrastructural changes in their mitochondria, which assume the condensed form [25]. Consequently, it has been speculated that in hyperthermic cells a correlation exists between changes in their energy metabolism and increased antitumour activity of lonidamine [25]. This point will be discussed later with respect to the mechanism of action of these compounds.

Preliminary studies also indicate that lonidamine inhibits the repair of potentially lethal damage produced by X-irradiation, methyl methane sulphonate and bleomycin [40]. A typical graph is shown in *Figure 3.3*.

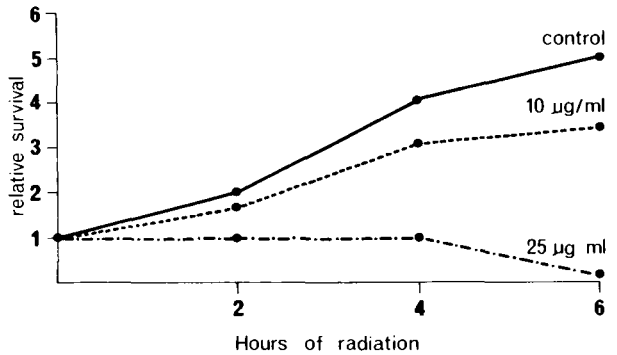


Figure 3.3. An in vitro *study of the effects of lonidamine combined with X-rays on the survival time of Chinese hamster cells (radiation = 1200 rads).*

Lonidamine inhibits recovery from potentially lethal damage at concentrations which are inactive when the drug is given alone. Since lonidamine is effective when added to these cultures after X-irradiation, it does not act as a radiosensitizer. It is known that recovery from potentially lethal

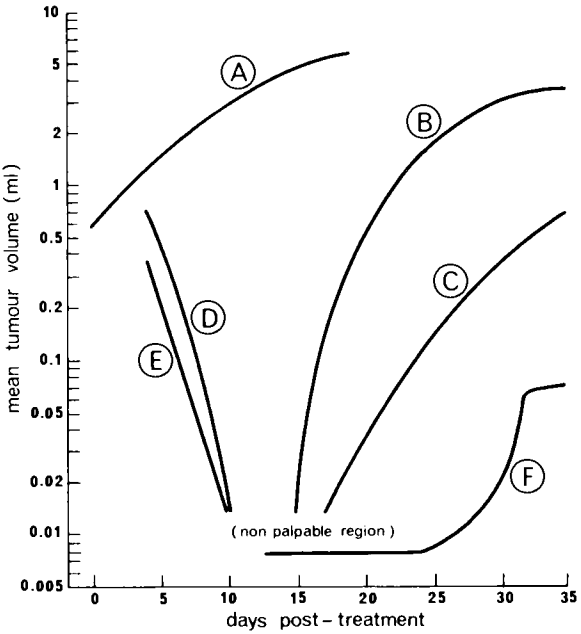


Figure 3.4. An in vivo *study of the effects of lonidamine combined with X-radiation on the METH/A tumour. (A) Control; (B)–(D) radiation alone; (C)–(E) radiation + lonidamine; (F) radiation + lonidamine (repeated treatment).*

damage is an energy-requiring process; therefore, these findings are consistent with the hypothesis that lonidamine affects the cell energy process.

The effects of the lonidamine combination with X-rays were also studied in mice inoculated with METH/A cells. The compound was administered both before and after exposure to X-rays in order to detect the eventual presence of a radiosensitizing action [37]. The most significant data are shown in *Figure 3.4.*

Lonidamine potentiates the effects of X-rays. This potentiation occurs even when the drug is administered afterwards, thereby confirming that it has no radiosensitizing activity. It is noteworthy that the effects of lonidamine were not detected on the immediate response to X-rays, but on the delayed recovery of the tumour.

The other compounds of this class have shown a similar pattern of antitumour activity, but are generally less active than lonidamine. For instance, the ratio of active doses of AF 1312/TS and lonidamine on Sarcoma 180 is about 10 : 1 [25].

The second characteristic activity of indazolecarboxylic acids is their antispermatogenic action. Since the pattern of this activity is similar in all these compounds, except for active doses, for convenience sake, we shall refer only to a few representative members of this series, namely, AF 1312/TS, lonidamine and tolnidamine (*Figure 3.1*) [13–17,24,28,41–49]. In rats, an antispermatogenic effect is observed within a few hours following a single oral administration. The antispermatogenic doses are devoid of other pharmacological effects and are consistently lower than those producing general toxicity [35]. With lonidamine and tolnidamine, the ratio between the antispermatogenic and toxic doses is around 1 : 100.

Both the Sertoli cells and the immature germ cells appear to be the target of indazolecarboxylic acids in the rat [16,42,49]. The first lesions are observed in the Sertoli cells and consist in apical retraction and vacuolation of the cytoplasm, after which they gradually spread to the spermatids. Subsequently, the Sertoli cells normalize, while the lesions of the spermatocytes become more evident. The spermatids are also affected. Five days following administration of lonidamine, new changes take place in the Sertoli cells which seemingly depend on the emptying of the seminiferous tubules rather than on a direct effect on these cells.

The fact that the first effects of indazolecarboxylic acids are produced on the Sertoli cells would suggest them to be the target of these compounds. This interpretation is also supported by the observation that androgen-binding protein (ABP) concentrations in the serum, testis and epididymis decline rapidly following lonidamine or tolnidamine administration [46]. In fact, ABP is produced by the Sertoli cells. In different animal species, the picture of the antispermatogenic activity of indazolecarboxylic acids is, however, substantially different. In dogs, lonidamine affects the more mature germ cells, i.e., the spermatids, without affecting the spermatocytes, as occurs in rats, even after 9 weeks of treatment with 25 mg/kg per day p.o. [43]. Moreover, in a preliminary study in man with lonidamine, a consistent reduction of spermatozoa in the ejaculate, without testicular shrinkage, occurred within 2 weeks from the beginning of treatment [50]. If confirmed, these data would demonstrate a direct effect on the more mature germ cells. It should also be pointed out that lonidamine produces on isolated germ cells of rats morphological lesions similar to those observed *in vivo* [29]. These findings are not in agreement with the hypothesis that the antispermatogenic action is the result of a primary effect on the Sertoli cells; at least, such a mechanism would not account for all the effects of these compounds on germ cells.

Histological studies have also shown that the Leydig cell is not affected; this finding is in agreement with the observation that the testosterone and luteiniz

ing hormone (LH) levels are unchanged in rats [46]. Moreover, the antispermatogenic activity of lonidamine and tolnidamine takes place before any change in follicle-stimulating hormone (FSH) concentrations occurs [46].

Apart from the genital system, no organ toxicity was detected in rats, even after daily administration of 400–800 mg/kg for 1 month [35]. These data suggest that a highly specific mechanism is involved in the antispermatogenic activity of indazolecarboxylic acids.

On account of their unique feature, indazolecarboxylic acids appear promising candidates as male contraceptives. For this purpose, tolnidamine seems the most preferable compound of this series because its toxicity is low, whereas its antispermatogenic activity is comparable with that of lonidamine.

The embryotoxicity of indazolecarboxylic acids will be discussed briefly, as relatively few studies are available to date. It is preferentially manifested at a specific stage of embryofœtal development, which in rats is the 9th day of pregnancy [26]. The minimal effective doses of AF 1312/TS and lonidamine are about 100 and 10 mg/kg p.o., respectively. This is the same potency ratio as for the antispermatogenic and antitumour activity [24].

BIOCHEMISTRY

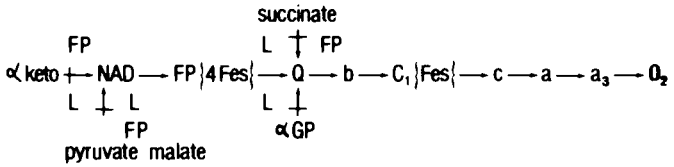
Spermatogenesis is accompanied by morphological changes of germ cell mitochondria consisting in a gradual passage from the orthodox to the condensed form [51–56]. These changes are accompanied by functional modifications consisting in an increase of oxidative capacity and phosphorylation [53]. The germ cells with condensed mitochondria are those susceptible to lonidamine and its active analogues. This observation suggested that the biochemical target of lonidamine and its active analogues might be the metabolically active stage of the mitochondrion, generally referred to as condensed mitochondrion.

This hypothesis has been corroborated by a number of observations. Lonidamine and its active analogues inhibit germ-cell respiration both *in vitro* and following oral administration; this effect is accompanied by an increase of aerobic glycolysis [22,29,30]. The antispermatogenic activity of these compounds is characterized by early changes of germ-cell mitochondria. In different members of this series, the potency of these effects is closely correlated to the potency of the antispermatogenic activity [30]. Lonidamine also produces early and specific ultrastructural changes in tumour cell mitochondria, accompanied by a decrease in respiration; unlike germ cells, in tumour cells there is also a decrease in glycolysis [22,29,47,57]. Inhibition of glycolysis

in tumour cells may be attributed to the presence of a mitochondrially bound hexokinase which is usually absent in normal, differentiated cells [57]. Consequently, both the effects on respiration and glycolysis are seemingly related to a primary effect on the mitochondrion.

As far as the inhibition of oxygen consumption is concerned [58], lonidamine has been shown to be more effective when the mitochondria are in the metabolically active state, namely, when the electron carriers are in an oxidized state. This occurs only when the mitochondria are in state 3, i.e., when adenosine triphosphate (ATP) is synthesized, or in uncoupled state 3. In fact, oxidation of nicotinamide adenine dinucleotide (NAD) and FAD-linked substrates is inhibited only when respiration is stimulated by ADP and carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP). Worth noting is that inhibition in the presence of FCCP is considerably higher than with ADP stimulated respiration, thereby confirming that the inhibitory effect may be correlated to the redox state of the respiratory chain [58].

The different sensitivity to lonidamine of NAD-linked substrates (pyruvate + malate, α-ketoglutarate) as compared with FAD-linked substrates (succinate) is also of interest. In all probability, this difference is due to a low degree of accessibility of the succinic dehydrogenase to the drug in that the addition of the non-ionic detergent Lubrol, which alters mitochondrial membrane permeability, brings the inhibition of succinate oxidation to similar values as those observed for pyruvate + malate. As far as lonidamine's site of action is concerned, it has been established that it does not affect the respiratory chain per se, but rather acts at the level of dehydrogenases as illustrated in *Scheme 3.3.*



Scheme 3.3.

Yet another point to be considered is that lonidamine acts only on intact mitochondria, being totally inactive on isolated dehydrogenase. In fact, lonidamine is nonspecifically bound to inert mitochondria and maximum binding is reached after 30 s. If the mitochondria are in state 4, i.e., in the absence of ADP, the affinity binding sites for lonidamine are low, whereas

high-affinity binding sites appear when ADP is added and oxygen consumption is inhibited [59]. This demonstrates that to be sensitive to lonidamine the mitochondria should be in a specific functional state. This state may be represented by the transformation from the orthodox to the condensed form of the inner membrane matrix compartment or the passage of dehydrogenases from a reduced to a more oxidized state or from a decrease of the ATP/ADP ratio.

To conclude:

(a) Lonidamine inhibits oxygen consumption in intact mitochondria and is totally inactive on isolated enzymes.

(b) Mitochondria possess two types of binding site for lonidamine – both low- and high-affinity. High-affinity sites appear when the mitochondria are metabolically active.

(c) Inhibition is not exerted at the level of the respiratory chain; lonidamine rather affects electron transport by blocking dehydrogenase.

(d) This takes place only when the mitochondria pass from the 'orthodox' to the 'condensed' form and/or when electron transport is in an oxidized state.

It may be also hypothesized that drugs and/or treatment which modify the energy status of mitochondria may enhance the effect of lonidamine. This is in agreement with experimental observations presented above.

The condensed mitochondrion may be considered a functional adaptation which is found in some tissues, such as tumours, seminiferous tubules and some stages of embryofœtal development, in which high energy requirements are combined with low oxygen tension [24]. Therefore, biochemical data provide a tentative explanation for the characteristic effects of indazolecarboxylic acids, suggesting that they depend on a common mechanism of action.

TOXICOLOGY

Indazolecarboxylic acids have a low acute toxicity, with LD_{50} values in rodents usually exceeding 1 g/kg p.o. [35]. It has already been mentioned that the acute toxicity of these compounds shows no correlation with the potency of their characteristic pharmacological effects. This is illustrated in *Figure 3.5*, in which the antispermatogenic activity and the acute toxicity of three representative derivatives are compared [24]. Interestingly, AF 1312/TS, which is the least active, is the most toxic.

Table 3.3 shows the most characteristic effects produced by high doses of lonidamine; these effects are essentially similar for most of the derivatives [27].

Lethal effects are produced within 24–48 h following administration and are

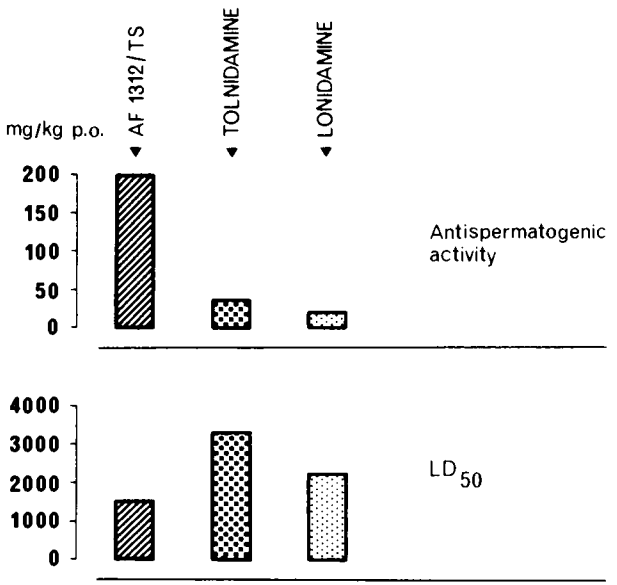


Figure 3.5. Quantitative correlation between antispermatogenic activity and acute toxicity of three indazoles.

Table 3.3. ACUTE EFFECTS OF INCREASING DOSES OF LONIDAMINE IN THE MOUSE AND RAT [27]

Species	*Dose (mg/kg)*	*Route*	*Effects observed*
Mouse	300	i.p.	sedation, analgesia and marked motor incoordination;
	675	p.o.	death of 10–20% of the animals
	450	i.p.	prostration followed by convulsive jerks; death of 40–50%
	1012	p.o.	of the animals
Rat	300	i.p.	sedation, analgesia, motor incoordination and rigidity of
	675–1012	p.o.	limbs
	450	i.p.	prostration followed by convulsive jerks; death of 30–40%
	1518	p.o.	of the animals

accompanied by general prostration, eventually with superimposed convulsions. This would suggest that death is due to pharmacodynamic effects rather than organ toxicity.

In the rhesus monkey, oral administration of 500 mg/kg of lonidamine for three consecutive days did not produce lethal effects, but a clinical symptomatology characterized by diarrhœa and vomiting. Blood tests showed leucocytosis with neutrophilia, increase in inorganic phosphorus, blood urea nitrogen (BUN) and serum glutamic-oxalacetic transaminase (SGOT). Post-mortem examination showed necrosis of the proximal convoluted tubules [45].

In studies with repeated administration [35] in the rat, lonidamine up to oral doses of 800 mg/kg daily for a month gave rise only to changes in the seminiferous epithelium in males. In rhesus monkeys, treated for the same length of time with doses up to 100–200 mg/kg p.o., renal disturbances were observed of the same type as in the acute studies. There were no deaths. Lonidamine was well tolerated in dogs treated for 2 months with doses of 25 mg/kg p.o., only spermatogenesis being affected by the treatment. Mutagenicity studies showed lonidamine to be devoid of genotoxic effects [36].

When compared with lonidamine, tolnidamine has been shown to have a similar toxicological profile, but is considerably less toxic.

Apart from embryotoxicity, which has been described among the pharmacological properties of these compounds, and their antispermatogenic effects, the potential toxicological hazards of indazolecarboxylic acids in rodents are very slight. In agreement with pharmacological data, this confirms that these compounds are very specific in their effects. In monkeys, instead, indazolecarboxylic acids present a risk of nephrotoxicity following both acute and chronic administration. This effect is accompanied by changes in serum chemistries, consisting mainly in acute rises in BUN which are normalized upon discontinuance of treatment. Therefore, the risk of kidney toxicity in man can be verified by routine laboratory tests. The fact that kidney toxicity in monkeys, produced only at relatively high doses, is dose-related, makes it reasonable to assume that at the recommended therapeutic doses this risk in man is low.

PHARMACOKINETICS

Lonidamine, tolnidamine and AF 1312/TS are the only indazolecarboxylic acids which have been studied from the point of view of pharmacokinetics [14,25,60–66]. Since their pharmacokinetic pattern is similar, only lonidamine, which has been studied the most, will be described.

Lonidamine was determined fluorimetrically [62], by HPLC chromatography [67] and using the tritiated compound [68–71]. Studies were performed in mice, rats, rabbits, monkeys and man.

In rats [66], lonidamine has a high oral absorption, as shown by the fact that the areas under the blood concentration curves following oral and intravenous administrations are similar. At the dose of 17 mg/kg p.o. which produces an antispermatogenic effect, blood levels are in the order of 20 μg/ml. The protein-binding capacity of serum is very high, in the order of 400 μg/ml. The half-life of lonidamine in the blood is 4.5 and 9.5 h at doses of 35 and 500 mg/kg p.o., respectively. Available data indicate that lonidamine is not degraded biologically and is eliminated unchanged or in the form of conjugated derivatives which are easily hydrolysed. Consequently, the use of the labelled compound provides valuable information on the drug itself. The distribution of radioactivity in various organs following administration of tritiated lonidamine indicates no preferential accumulation, apart from that in the excretory organs (kidney and liver). The compound is excreted mainly in the fæces, only a small amount being present in the urine.

In monkeys [66], according to the above-mentioned criterion of comparing areas under the blood concentration curves, oral absorption is approximately 50% of the administered dose. The dose of 50 mg/kg p.o. produces blood levels in the order of 10–20 μg/ml. Urinary excretion in this species is very high. Following administration of 50 mg/kg p.o., about 40% of the dose is found in the urine.

In man, lonidamine has been studied in patients with cancer, usually at a very advanced stage. A single administration of 300 mg produces blood concentrations averaging 6–20 μg/ml, with large variations from patient to patient [60,72]. It should be noted that the above concentrations are in the same range as those found in mice receiving antitumour doses [25]. Preliminary results, obtained from a small patient population, showed a correlation between blood levels and antitumour activity [60]; this correlation, however, was not confirmed by subsequent studies [73].

In man, lonidamine is excreted mainly in the urine, in the free or conjugated unchanged form [60,65]. The oral absorption of lonidamine in man cannot be accurately assessed in that no comparison has been made with intravenous administration. Nevertheless, the blood levels and urinary excretion after oral administration suggest that the drug is well absorbed orally.

CLINICAL INVESTIGATIONS

This review is limited to the clinical study of lonidamine in cancer patients; the antispermatogenic activity of some indazolecarboxylic acids was also studied, but results to date are too preliminary to draw any conclusion.

The study of lonidamine in cancer patients began in 1980 at the F. Angelini Oncology Centre, Ancona [74]. Side-effects and maximum tolerated doses were investigated, and a standard dosage scheme was established. The effects of lonidamine in advanced brain, breast, lung and other typical tumours were also studied, using the drug both alone and in combination with some chemotherapeutic agents. While this study was in progress, a number of systematic clinical trials were undertaken in Europe and North America. Although still incomplete, these studies, on the whole, provide a sound indication of the potentiality of lonidamine in the field of cancer. Results of clinical trials on lonidamine may be summarized as follows [74–78].

Lonidamine was given orally for 1–60-day periods. Doses ranged from 90 to 520 mg/m^2, divided in two or three daily administrations. Toxicity consisted mainly of myalgias, gastrointestinal complaints and testicular pain and, less frequently, anorexia, somnolence, photophobia, impaired hearing of high-pitched sounds, skin hyperesthesia and asthenia. Side-effects tended to disappear with time without interruption of treatment. The mechanism responsible for myalgias, the most common side-effect of lonidamine in cancer patients, has not yet been elucidated. It has been reported that the administration of prednisone, 5 mg b.i.d., suppresses or reduces myalgias [65].

No laboratory abnormalities related to drug treatment have been reported, but occasionally an increase in serum lactic acid and a fall in serum testosterone levels have been described [76,78]. The latter finding is in contrast both with animal data [46] and with results obtained in healthy volunteers after 7 days of treatment [79].

Broad spectrum clinical trials are in progress in a variety of advanced and previously treated tumours [75,76,78]; these include colorectal carcinoma, breast cancer, small cell lung cancer, non-small-cell lung cancer, soft tissue sarcoma, renal carcinoma, melanoma, hepatoma, germ-cell tumour of mediastinium and histiocytoma. Lonidamine is being given at daily oral doses ranging from 270 to 520 mg/m^2, subdivided into two or three administrations. Results so far show partial responses in four patients (two with breast cancer, one with synovial sarcoma and one with hypernephroma). Disease-oriented clinical studies are also in progress in cases of colorectal carcinoma, brain tumour, chronic lymphoid leukemia and previously untreated, unresectable non-small-cell lung cancer.

No responses were reported in any of the patients with colorectal carcinoma treated with lonidamine [76]. Two out of nine patients with brain tumour, previously submitted to surgery and radiotherapy, improved with lonidamine [80].

Of 19 patients with chronic lymphoid leukaemia, two showed a significant decrease (to less than 70% of the pretreatment values) in the lymphocyte count and three patients showed an appreciable reduction of the spleen or lymph-node size [81]. Six partial and seven minor responses, 14 of no changes (for at least 1 month) and 12 progressions out of 39 assessable patients with non-small-cell lung cancer were observed [82].

Further studies are in progress in breast and lung cancer, renal carcinoma, soft-tissue sarcoma and cancer of the prostate.

Since laboratory data show that some tumour systems become sensitive to lonidamine following exposure to hyperthermia, radiation and alkylating agents [25,32,33,37,39,40], clinical studies were undertaken to investigate the effects of lonidamine combined with chemotherapy [74,83,84] and radiotherapy [85]. Some preliminary results are available suggesting that chemotherapy and radiotherapy potentiate the activity of lonidamine.

To date, the best results have been obtained in patients with brain metastasis of primary lung or breast tumours [74] receiving lonidamine combined with carmustine (BCNU) and procarbazine (20 complete or partial responses out of 30 assessable patients). Good results have also been reported in head and neck cancer [85] with lonidamine combined with radiotherapy (26 complete responses out of 40 cases, as compared with 17 out of 50 cases observed in a comparable historical control treated with radiation alone).

Randomized, double-blind studies are underway.

CONCLUDING REMARKS

Interest in indazolecarboxylic acids was raised by the unexpected observation that some of them possess an antispermatogenic activity in animals. This finding suggested the potential usefulness of these compounds as male contraceptives. Nevertheless, this objective has somewhat been neglected during the last years and priority has been given to basic research in an attempt to clarify the mechanism of action of these compounds. There were two reasons for this. First, the antispermatogenic action of indazolecarboxylic acids is remarkably specific. Not only is it produced by doses much lower than those possessing measurable pharmacological or toxicological effects, but chemical manipulations of the basic structure increase the antispermatogenic activity without

any parallel change in general toxicity; since indazolecarboxylic acids do not accumulate in the testis, there is an indication that they might affect a biological mechanism playing a role in spermatogenesis, but not in other cell systems. Hence, the idea that the study of indazolecarboxylic acids could provide an insight into a new biological mechanism. Second, since contraceptives are intended for healthy people, the problem of their safety was considered to be of the utmost importance; in our opinion, no drug can be considered safe unless its mechanism of action is known.

The decision to give priority to basic research had some unexpected consequences. From a practical viewpoint, the most important one was the discovery that some indazolecarboxylic acids possess antitumour activity. Initially, this activity was overlooked because of the impossibility of detecting it in the standard screenings (such as the NCI test) for antitumour activity. Incidentally, it should be recalled that these screenings were set up for agents affecting primarily cell division processes; the experience with indazolecarboxylic acids raises the question as to whether or not the current animal models of tumours are suitable for detecting anticancer agents with different modes and mechanisms of action. It was also discovered that indazolecarboxylic acids possess a selective embryotoxic activity. These findings, together with a number of biochemical and ultrastructural observations, led to the working hypothesis that spermatogenesis, some tumour systems and a stage of embryofœtal development use a specialized energy-yielding process; this process was hypothesized to be the target of indazolecarboxylic acids.

Biochemical studies have shown that indazolecarboxylic acids inhibit germ-cell respiration, whereas glycolysis is increased. During spermatogenesis, germ-cell mitochondria present some well-known and characteristic morphological and functional changes, leading to the formation of the so-called condensed mitochondrion. The current hypothesis is that this is the target for the indazolecarboxylic acids. Both respiration and glycolysis are inhibited in cancer cells but not in germ cells, treated with indazolecarboxylic acids. This phenomenon is explained by the existence, in cancer cells, of a mitochondrially bound hexokinase; consequently, the speculation that in both germ and cancer cells the target of indazolecarboxylic acids is the mitochondrion provides a reasonable explanation for the different biochemical effects of these compounds in germ and cancer cells.

Indazolecarboxylic acids inhibit both respiration and glycolysis in cancer cells, whereas in germ cells only respiration is inhibited and a compensatory increase of glycolysis is observed; despite this, their antispermatogenic activity is far more regular than the antitumour one. It can be speculated that the so-called condensed mitochondrion occurs spontaneously during spermato

genesis, whereas it develops in cancer cells only in conditions of increased energy demand. This speculation is of practical interest, since it postulates that conditions or factors capable of increasing the energy demand of cancer cells may also increase their response to indazolecarboxylic acids.

Present studies are focused on the therapeutic activity of lonidamine in cancer conditions and in their use as a tool to investigate some specialized energy-yielding processes. Available data indicate that lonidamine is of potential usefulness in cancer therapy alone or combined with other agents which increase the response of the cancer cells to lonidamine. Information on the mechanism of action of indazolecarboxylic acids has also renewed interest in their potential use as male contraceptives; in this connection, the most promising derivative of the series is tolnidamine.

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4 Monoamine Oxidase Type B Inhibitors in the Treatment of Parkinson's Disease

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INTRODUCTION

Parkinson's disease (PD) is associated with a deficiency of dopamine (DA) in the extrapyramidal brain regions [1]. Treatment with levodopa (L-DOPA, 3,4-L-dihydroxyphenylalanine) is considered to be a DA replacement therapy [2]. Many of the clinical problems besetting present-day therapy of PD derive from centrally induced adverse reactions to moderately large doses of levodopa. Chronic treatment with levodopa increases the incidence of side-effects, including increased occurrence of 'on-off' phenomena, and the therapeutic response diminishes with time [2]. These changes can be explained by: (a) diminution of the uptake of levodopa by the degenerated dopaminergic neurons; (b) rapid deamination of DA (formed by the decarboxylation of levodopa) by monoamine oxidase (MAO) and catechol *O*-methyltransferase (COMT); (c) desensitization of the dopaminergic receptor; and (d) inhibition of the firing of the DA neurone by accumulating metabolites [1,3,4].

In the past few years, a number of approaches have been made in the development of drugs for the treatment of PD. There is ample evidence to suggest that there are at least two subtypes of dopaminergic receptor, D_1 and D_2, and it has been proposed that most of the desirable dopaminergic effects arise from activation of D_2 receptors in the striatum which contribute to a therapeutic response to levodopa or DA agonists such as bromocryptine (see Ref. 5 for review). Although DA agonists have been used in the treatment of PD, especially in patients who either do not respond to levodopa or develop severe 'on-off' effects, the adverse side-effects of these drugs outweigh their anti-Parkinsonian property [5–8].

Another logical approach in the development of anti-Parkinson drugs is the pharmacokinetic manipulation of DA formed from levodopa using either MAO [2,9] or COMT [10] inhibitors. This approach would be suitable if the 'on-off' phenomenon and loss of levodopa therapeutic response are due to lack of an adequate supply of DA or to the accumulation of DA metabolites [3]. At present, the inhibitors of COMT have proven to be unsatisfactory in the treatment of PD [11]. But recent developments in the biochemistry and pharmacology of MAO and its inhibitors would strongly indicate that there is a place for MAO inhibitors as adjuncts to levodopa therapy of PD. This chapter is in no way intented to be a comprehensive review of MAO [12,13], its inhibitors [12] or PD [2], but we wish to discuss the rôle of one class of MAO inhibitors, namely, selective MAO type B inhibitors, which have in the past 10 years proven to be successful in the treatment of 'on-off' effects and recouping of the loss of therapeutic response in levodopa-treated Parkinsonian patients.

BRAIN MAO

Brain has half the MAO activity of liver [14]. This is true both for rat as well as for human tissues. The enzyme in brain is unevenly distributed and the highest activities are found in hypothalamus and striatum [15], low activity being associated with the cerebellum and cerebral cortex. More than 70% of activity is found in the mitochondrial fraction and all of this activity is associated with the outer mitochondrial membrane [16] in both rat and human brain [17].

Intraneuronal as well extraneuronal MAO is responsible for the oxidative deamination of a variety of monoamines, among which are the well known neurotransmitters DA, serotonin (5-HT) and noradrenaline (NA), as well as monoamines such as 2-phenylethylamine (PEA), tryptamine and octopamine [13,14]. It has long been known that intraneuronal MAO is responsible for maintaining a low concentration of the neurotransmitter in the cytoplasm, since when brain MAO is inhibited *in vivo* with irreversible MAO inhibitors, brain levels of the biogenic monoamines increase substantially [13,14] (see a later section).

MULTIPLE FORMS OF MAO AND THEIR FUNCTION

IN VITRO EVIDENCE

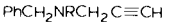
Initial propositions that MAO exists in multiple forms having different substrate specificities and inhibitor sensitivities were made by Youdim and Sourkes [17] and Squires [18]. Subsequently, it was shown that solubilized purified brain and liver MAO can be separated electrophoretically into a number of active bands having different substrate and inhibitor specificities [19,19a]. These latter studies were challenged by Tipton and Houslay [20], who suggested that the so-called multiple forms of MAO were artefacts of solubilization and electrophoresis. Later, Johnston [21] described an unusual action of the MAO inhibitor, clorgyline, which in *in vitro* studies could distinguish between at least two forms of the enzyme, termed 'type A' and 'type B'. Type A enzyme is responsible for deamination of hydroxylated amines (5-HT, NA, adrenaline and octopamine) and is selectively inhibited by the irreversible 'suicide' inhibitor, clorgyline, while the non-hydroxylated amines PEA, benzylamine, tryptamine, octylamine and decylamine [13,22] are substrates of the type B enzyme (which is resistant to inhibition by clorgyline [21,23]). DA and tyramine are considered to be substrates for both enzyme forms. The selective

Table 4.1. SELECTIVE INHIBITORS OF MAO TYPE B

a Irreversible inhibitors

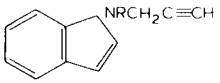
Clorgyline

Selegiline



Pargyline, R=Me

N Demethylpargyline, R=H

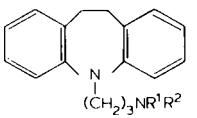


AGN 1133, R=Me

AGN 1135, R=H

(+)-Tranylcypromine

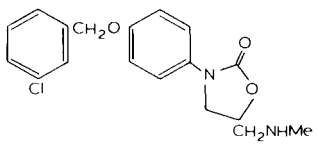
b Reversible inhibitors



Imipramine, $R^1=R^2=Me$

Demethylimipramine, $R^1=Me$, $R^2=H$

Didemethylimipramine, $R^1=R^2=H$



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inhibitors of type B MAO include selegiline ((−)-deprenyl [12,24], pargyline [12,23] and AGN 1135 [25,26]. Like clorgyline, these drugs also possess the acetylenic reactive group which interacts covalently and stoichiometrically with the MAO flavin adenine dinucleotide cofactor at the active site of the enzyme [14,27] (*Table 4.1*).

In the human brain, DA has been on various occasions described as being a substrate for the two forms of MAO [29–31] for the A type only [32,33] and for the B type only [18,34,35,36]. In animal brain studies, the same uncertainties also exist, since in the liver both enzyme forms metabolize DA [28,37] but MAO type A has been reported to be responsible for DA

deamination in the rat brain [38]. The latter data are in sharp contrast to the studies of Green, Mitchell, Tordoff and Youdim [39], who showed by both biochemical and animal behavioural studies that DA was a substrate for both enzyme forms in the rat brain. When rats were given a nonspecific inhibitor of MAO (e.g., tranylcypromine) followed by levodopa, the brain DA concentration rose significantly and the animals developed a syndrome of hyperactivity and stereotypity. The increase in brain DA was far greater than that of NA. However, when brain MAO types A or B were selectively inhibited with either clorgyline or selegiline and rats received levodopa, even though brain DA concentrations increased, they were much less than those seen after tranylcypromine and the animals did not develop hyperactivity. These results were interpreted as indicating that deamination of DA takes place *in vivo* by both enzyme forms. This conclusion received further support from the data obtained when animals were injected a combination of the two selective inhibitors prior to levodopa. The brain DA concentrations in these animals were as high as those seen with tranylcypromine and the animals developed the syndrome of hyperactivity [38].

Because of uncertainties concerning the distribution of MAO A and B in the human brain and the specificity of DA for the two enzyme forms, the deamination of DA, 5-HT and PEA was examined in seven human brain regions including substantia nigra and caudate nucleus [33]. In all brain regions, both A and B forms of MAO were found to be active towards DA. However, the ratio of activities of MAO A and MAO B were found to vary considerably in different brain regions. In the caudate nucleus more than 70% of DA was deaminated by MAO B, a result which confirms earlier reports [35] using tyramine as substrate. Thus, in this region the major form of MAO is type B.

The biochemical and pharmacological differentiation of MAO into two subtypes as suggested by Johnston [21] has been used to develop a large number of MAO inhibitors with selectivity towards each enzyme form [12]. There have been many investigations into the distribution and the ratio of MAO types A and B in a variety of tissues and species [28]. More pertinent to the present discussion is the presence and distribution of the two enzyme forms in brain, especially that of the human brain. Of special significance is the MAO activity in the extrapyramidal brain regions.

IN VIVO EVIDENCE

If the evidence gathered from *in vitro* studies truly indicates that MAO exists in different forms having properties that would selectively recognize specific

substrates, then *in vivo* studies should substantiate these results. There are numerous studies in which MAO activity has been measured in homogenates or mitochondrial preparations or in isolated perfused tissue preparations from animals pretreated with various doses of either clorgyline or selegiline. The results of these experiments clearly substantiate the *in vitro* results, namely that substrate-selective inhibition of MAO can be maintained *in vivo* in peripheral tissues as well in the brain [12, 40–45]. Furthermore, it has been shown that, while clorgyline administration in rats causes a rise in brain 5-HT, DA and NA, the concentration of PEA remains unchanged. The administration of the selective MAO B inhibitor, selegiline, results in an increase in DA and PEA only [45]. Thus, as far as the rat brain is concerned, DA is a substrate for both enzyme forms [33,40,42]. The apparent increases in rat brain 5-HT, NA or PEA after the administration of the individual selective inhibitors are not as large as those seen when a non-selective inhibitor (e.g., tranylcypromine) or the combined clorgyline and selegiline treatments are used [44,46]. These findings were interpreted as indicating that while 5-HT or NA are, under normal circumstances, deaminated exclusively by the type A MAO, when this enzyme form is selectively inhibited by clorgyline, brain 5-HT or noradrenaline concentrations rise to an extent that would satisfy the K_m of type B enzyme and these amines can then be deaminated by MAO B [46,47]. A similar phenomenon is also observed for brain PEA after the selective inhibition of MAO type B [48,49]. To a large extent, these results have been confirmed in *in vitro* studies using brain mitochondrial preparations [31,50]. Thus, it can be concluded that the multiple forms of MAO do not show strict selectivity towards either their substrates or inhibitors.

Further evidence that MAO types A and B show functional difference *in vivo* has come from the measurement of the deaminated metabolities of 5-HT, NA, DA and PEA in the rat brain and urine after selective inhibition of MAO with clorgyline or selegiline. While after the administration of clorgyline there was a substantial decrease in 5-hydroxyindoleacetic acid, methoxyphenylglycol, dihydroxyphenylacetic acid and homovanilic acid, selegiline treatment caused a reduction of dihydroxyphenylacetic acid, homovanilic acid and phenylacetic acid [51–54].

Although much controversy surrounds the biochemical basis of MAO multiple forms, the *in vitro* and *in vivo* studies clearly demonstrate that functionally the enzyme behaves as two distinct enzyme systems which can be selectively inactivated with drugs. Thus, it is apparent that the neurotransmitter levels in the brain can be selectively manipulated by the use of type A or type B inhibitors.

MAO TYPE B INHIBITORS AS DRUGS

CLASSES OF INHIBITOR

Both irreversible and reversible inhibitors have been developed, of which only the irreversible inhibitors have seen extensive clinical application. In recent years, reversible selective MAO type A inhibitors have been developed for use as antidepressants, and claims have been made that reversible inhibitors should be less toxic in clinical use than irreversible inhibitors. No major trend has appeared in the development of MAO type B reversible inhibitors for treatment of Parkinson's disease, and the justification of such an approach remains speculative.

Compounds which have received adequate description in the literature and have a good degree of selectivity for MAO type B are listed in *Table 4.1*.

(a) Irreversible inhibitors

For a more comprehensive review of the different types of MAO inhibitor available, see Refs. 13, 14. The MAO type B selective irreversible inhibitors so far described are either acetylenic or cyclopropylamine compounds. These compounds are 'suicide' inhibitors, since the initial stage in their action is conversion by the target enzyme into an active electrophilic compound which then combines covalently at the enzyme active site (see above).

With both type A and type B inhibitors, selectivity is relative and is lost as inhibitor concentration is increased. The type B inhibitors are also less selective for the type B enzyme than are the type A inhibitors for type A enzyme. Maintenance of selectivity for MAO type B inhibition *in vivo* on chronic administration, is, therefore, dependent on careful control of dosage.

Pargyline is one of the earlier MAO inhibitors, which was used for a considerable period of time as an antidepressant and antihypertensive agent. In isolated enzyme systems and in appropriate dosage *in vivo*, pargyline shows selectivity of inhibition for MAO B [25,55]. Inhibition of MAO *in vivo* is cumulative with irreversible inhibitors, and a dose of pargyline which shows selectivity for MAO B after a single administration may produce non-selective inhibition of both enzyme types when administered chronically [56]. A single dose of 30 mg in man produced selective inhibition of MAO type B, as shown by a more than 90% inhibition of platelet MAO B activity with a negligible reduction in plasma MHPG [57]. At this dose level, pargyline exerts no hypotensive action, but doses in the region of 90 mg/day produced nonspecific inhibition of both MAO types, together with significant hypotensive effect [58].

The *N*-demethyl derivative of pargyline is a less potent inhibitor but shows greater selectivity for MAO B [25]. A similar relationship between *N*-demethyl derivatives and parent compound is seen in the experimental inhibitors, AGN 1135 and AGN 1133. A greater selectivity for MAO type B with reduction in potency is shown by AGN 1135 as compared with AGN 1133 [25,59]. The compound AGN 1133 is one of the most potent inhibitors described and was synthesized independently by Knoll, Ecsery, Magyar and Satory [59] and by Huebner, Donoghue, Plummer and Furness [60]. This compound is a highly effective inhibitor of MAO B, both *in vivo* and *in vitro*, but with poor selectivity. AGN 1135 shows a good degree of selectivity both *in vivo* and *in vitro* in rat tissues and in human brain tissue *in vitro* [25, 36, 61].

The best-described MAO type B inhibitor, selegiline, is a propargyl derivative closely related to pargyline (*Table 4.1*). As with other compounds of this type, enzyme inactivation proceeds through an initial stage of reversible, competitive combination with the enzyme followed by irreversible, covalent bonding [62]. In the initial stage, amine compounds which are also substrates for MAO type B will compete with the inhibitor for the enzyme, and will, if present in sufficient concentration, prevent the subsequent stage of irreversible inactivation.

A number of MAO inhibitors show stereoselectivity (see Youdim and Tenne [63] for review). The (−)-isomer of selegiline is more effective as an MAO inhibitor, while the (+)-isomer shows greater effectiveness in release of catecholamines and inhibition of neuronal amine uptake. Conversely, (+)-tranylcypromine shows relative selectivity for inhibition of MAO B, while (−)-tranylcypromine is less effective as an MAO inhibitor, and more active in inhibition of neuronal uptake and release of amines than the (+)-isomer.

The (+)-isomer of tranylcypromine shows a slight preference for MAO type B *in vitro* using rat brain [64] and produces effective inhibition of the type B enzyme *in vivo*, as shown by inhibition of platelet MAO and increased PEA levels [36].

As a result of the chemical reaction between MAO inhibitor and enzyme, the inhibitor is metabolized to the parent amine. Thus benzylamine is a metabolite of pargyline [65] and both amphetamine and methamphetamine (15% and 63%, respectively, of administered drug) are produced following *in vivo* metabolism of selegiline [66]. Structure-activity relationships within the propargyl series of compounds have been described by Knoll, Ecsery, Magyar and Satory [59] and by Kalir, Sabbagh and Youdim [25]. For the propargyl derivatives, compounds with a short (1 or 2 C atoms) carbon side-chain between phenolic group and propargyl nitrogen atom show selectivity for MAO B, whereas in the selective MAO A inhibitors the side-chain is equivalent

to 3- or 4-carbon units. This relationship, however, is not apparent in other chemical types of MAO inhibitor.

(b) Reversible inhibitors

The tricyclic antidepressants have been shown to produce a selective reversible inhibition of MAO type B [67,68] but are not potent in this effect, and the significance of the finding for the therapeutic action of the drugs is doubtful. Chlorpromazine was of equal effectiveness to imipramine in inhibiting MAO B of rabbit brain. K_i values for inhibition of PEA deamination *in vitro* by imipramine and amitriptyline were 4×10^{-5} M and 5×10^{-6} M, respectively, showing that the potency of this effect is several orders of magnitude less than that for inhibition of amine uptake. It is of interest that the *N*-demethyl and *N*-didemethyl derivatives of imipramine were equally as effective as the parent compound in inhibition of MAO B.

Structure-MAO inhibitory activity relationships for a series of oxazolidinone compounds were described by Dostert, Strolin Benedetti and Jalfre [69]. One of these compounds, MD 780236, acts as a selective inhibitor of MAO type B both *in vivo* and *in vitro* [70]. When examined *in vitro*, MD 780236 produced irreversible inhibition of the enzyme, since inactivation of enzyme activity was not reversed by dialysis. When injected into animals, however, most of the enzyme activity had returned 24 h after administration. It was suggested that metabolism of an imine intermediate formed from MD 780236 or metabolism to a reversible inhibitor *in vivo* could account for its more rapid reversal *in vivo* than *in vitro*. This suggestion has recently been supported by experimental evidence [71].

PHARMACOLOGY OF SELECTIVE MAO B INHIBITORS

An important point in considering the pharmacology of MAO inhibitors is that, like other drugs, these substances possess intrinsic pharmacological activity in addition to their shared property of MAO inhibition. Tranylcypromine, for example, is an effective inhibitor of neuronal amine uptake [72] and has amphetamine-like properties [73]. These additional pharmacological properties may be reversible and short-lasting, as opposed to the irreversible, long-lasting effect on MAO inhibition.

Early reports on the pharmacology of selegiline showed that this drug did not potentiate, and even inhibited, the effects of tyramine in a number of pharmacological preparations [74] as opposed to the tyramine potentiation ('cheese effect') produced by non-selective MAO inhibition. Subsequently, it

was shown [75] that the pressor effect of orally administered tyramine in human volunteers was not potentiated by a dose of selegiline which is adequate for selective inhibition of MAO type B activity.

In most of the animal studies, potentiation of sympathomimetic amine has not been correlated with degree of MAO inhibition, and in many acute, pharmacological studies, doses of inhibitor are used in excess of those used clinically, in order to obtain a satisfactory degree of MAO inhibition in a short period. At these higher dose levels of inhibitor, additional pharmacological effects may be seen which do not appear at lower doses of the drug.

The non-potentiation of tyramine effects by selegiline was attributed by Knoll to several factors: (a) selegiline does not inhibit intestinal MAO in man, which is of type A; (b) selegiline blocks neuronal amine uptake; and (c) selegiline blocks release of noradrenaline from storage vesicles [24,74].

The blockade of tyramine response in isolated tissue preparations is seen only at concentrations above 10^{-5} M, and in the whole animal, inhibition of tyramine pressor response was noticed at doses above 5 mg/kg [24]. Similarly, the effect of selegiline on vascular noradrenaline release and inhibition of amine uptake [76] was found only at high concentrations of the drug.

The effect of clorgyline and selegiline on inhibition of DA, NA and 5-HT uptake has been investigated in rat brain synaptosomes [77]. Selegiline was most efficient in inhibition of NA uptake (IC_{50} value = 26 μM) and less effective against 5-HT and DA uptake (IC_{50} value = 46 μM and 53 μM). In a similar study, the EC_{50} value for selegiline in inhibition of ^{3}H-labelled noradrenaline uptake in rat brain synaptosomes (10^{-6} M) was 10,000-fold higher than that of demethylimipramine [78]. The ability of selegiline to inhibit NA uptake *in vivo* was investigated by Simpson [79]. A dose of 10 mg/kg inhibited [^{3}H]noradrenaline uptake in rat heart by about 50%, although complete blockade of MAO B is achieved with 1 mg/kg [40,44].

Finberg, Tenne and Youdim [26] investigated tyramine antagonistic properties of selegiline and AGN 1135 in the isolated rat vas deferens preparation. Both compounds antagonized tyramine responses at concentrations of 10^{-5} M, but tyramine responses were potentiated on washing the inhibitors out of the organ bath. At a concentration of 10^{-5} M, selegiline reduced [^{3}H]metaraminol uptake in the vas deferens by only 20% and had no effect on [^{3}H]octopamine uptake. In the denervated vas deferens preparation, it was shown that selegiline possesses non-competitive α-adrenoceptor antagonistic properties. Concentrations of selegiline which are selective for MAO type B inhibition in isolated tissues did not cause any modification of tyramine response. In conclusion, the reversible tyramine antagonistic property of both selegiline and AGN 1135 in isolated tissues is seen only at high concentrations of the inhibitors, and may

be explained mainly by α-adrenoceptor antagonism or a nonspecific effect on smooth muscle contractility. A comparable effect of selegiline on tyramine pressor responses, however, is not observed in the whole animal, since tyramine effects are not reduced even at high doses of selegiline. In the whole animal, however, metabolism of selegiline to amphetamine and methamphetamine must be considered.

It may not be necessary to look for a pharmacological mechanism for the lack of 'cheese-effect' by selegiline, in view of the fact that neuronal MAO may be mainly type A. Evidence for the neuronal localization of MAO type A comes mainly from experiments involving sympathetic denervation of a variety of tissues, which produces a selective reduction of type A MAO activity in the tissue [51,80]. Tyramine, an indirectly acting sympathomimetic amine, must be taken up by sympathetic neurones in order to release noradrenaline, which produces the effect at the adrenoceptors. Release of noradrenaline by tyramine is not by an exocytotic process, but by displacement into the neuronal cytoplasm, so that such displaced noradrenaline is partially metabolized by MAO before egress from the neurone [81]. It follows, therefore, that inhibition of neuronal MAO would produce a profound effect on tyramine responsiveness by (a) reducing metabolism of tyramine within the neurone, and (b) protecting released noradrenaline from metabolism by MAO within the neurone. Evidence for this assumption was produced by Simpson, who showed that the sympathomimetic action of amphetamine (an indirectly acting amine, itself not a substrate for MAO) was potentiated by clorgyline but not by selegiline [82].

The above-described mechanism will result in potentiation of the effects of tyramine by an MAO inhibitor, even without alteration in systemic tyramine metabolism. In the clinical context, however, 'cheese-effect' is comprised of a reduction in extraneuronal metabolism, and elevated blood levels of tyramine, in addition to the neuronal mechanism. Orally administered tyramine is metabolized in both gastrointestinal tract and liver before entering the systemic circulation. Since MAO activity in the gut is mainly type A [18], the lack of inhibition of this enzyme activity by selegiline is one reason for the non-potentiation of tyramine effects by the selective MAO B inhibitor. Selective MAO type A inhibition using MD 780515 in the dog [83] or clorgyline in the cat [84] enhanced tyramine absorption from the gut, whereas selegiline did not. Hepatic MAO activity in man is comprised of a similar proportion of type A and type B enzyme, and so inhibition of either form would be anticipated to cause a similar reduction in tyramine metabolism. The effect of hepatic MAO B inhibition on systemic tyramine levels is apparently insignificant, as shown by the lack of tyramine potentiation by selegiline.

In doses slightly higher than those necessary for selective MAO B

inhibition, selegiline produces sympathomimetic effects in cat [85] and rat [79] as shown by contraction of nictitating membrane (cat) and increased blood pressure and heart rate (rat). At present, it is not clear whether these sympathomimetic effects are caused by the intact selegiline molecule or by its metabolites; they are, however, dependent on release of noradrenaline, since they are antagonized by reserpine or 6-hydroxydopamine pretreatment [79] and are absent following sympathetic denervation [85]. Demethylimipramine also antagonized the pressor effects of selegiline [79], which was taken to indicate that selegiline is a substrate for the neuronal amine uptake system. An alternative explanation for this effect is that demethylimipramine antagonizes the active efflux of noradrenaline following its release by the indirectly acting amine. Such an active efflux system was proposed by Paton [86] to participate in the action of indirectly acting amines such as tyramine and phenylethylamine.

Metabolism of selegiline yields the (−)-isomers of amphetamine, which are about 10-fold less effective in behavioural stimulation than the (+)-isomers. Metabolism to (+)-amphetamine isomers may be important in the effects of (+)-selegiline described in earlier publications [24,76]. The role of (−)-amphetamine in the pharmacological action of selegiline is at present not clear. In the *in vitro* system of superfused rat striatal tissue, selegiline (10^{-5} M) had no effect on [^{3}H]dopamine release [87], but the effect of selegiline on dopamine release *in vivo* may be different.

In rats with 6-hydroxydopamine-induced lesion of the medial forebrain bundle, selegiline was effective in producing ipsilateral circling, even at the relatively low dose of 1 mg/kg [88]. Such behaviour is also produced by amphetamine, and is ascribed to activation of striatal dopamine receptors [89]. Stimulation of circling by selegiline was reduced by administration of a dopamine β-hydroxylase inhibitor, which indicates that this deprenyl effect may be mediated partly by noradrenaline release. Selegiline also potentiated levodopa-induced circling in similarly lesioned rats [90]. It would be of interest to investigate whether another selective MAO B inhibitor, devoid of amine-releasing properties, also stimulates circling in rats with lesions of the nigro-striatal pathway. Little or no amphetamine-like behavioural stimulation was seen, however, in intact mice or rats injected with selegiline [91] and no effect on conditioned avoidance response was produced in rats with unilateral lesions in the substantia nigra [92]. In spite of the sympathomimetic effects of selegiline in laboratory animals described above, the administration of selegiline to humans is not accompanied by any reports of hypertensive crisis, apart from the mention by Yahr of one patient who appeared to have a hypertensive response [90].

Not only tyramine, but in fact all indirectly acting amines are potentiated by

MAO inhibition. Of these amines, those that are selective substrates for MAO B may be potentiated by selective MAO type B inhibitors by virtue of inhibition of their extraneuronal metabolism. All indirectly acting amines, even those which are selective substrates for MAO type B, will be potentiated by MAO type A inhibitors, because of inhibition of the neuronal MAO. PEA is an indirectly acting amine which is a MAO type B substrate. The action of PEA in contraction of cat nictitating membrane [93] and elevation of blood pressure [94] is markedly potentiated by selegiline treatment. The pressor effect of PEA in the cat was also potentiated by clorgyline [94], in keeping with the expected effects of inhibition of neuronal MAO. Behavioural effects of PEA in the rat were enhanced by selegiline but not by clorgyline [78], but behavioural effects of PEA in the monkey were enhanced by both clorgyline and pargyline, in doses which would be expected to inhibit selectively MAO A and B respectively [95]. In central as well as peripheral systems, the effect of selective MAO inhibition on metabolism of PEA itself as well as on metabolism of dopamine and noradrenaline release by PEA must be considered. Endogenous levels of PEA are elevated in brain of man following administration of selegiline [96] and urinary excretion of PEA is sharply increased at the start of selegiline therapy [75]. It has been suggested that this 'trace amine' could play a role in the etiology of Parkinson's disease and affective disorders and in the pharmacological response to MAO inhibitors [97]. Both these conditions have been associated with a reduced urinary excretion of PEA, but determination of urinary PEA excretion is subject to wide variability and considerable methodological difficulties [98].

In addition to the facilitation of central dopaminergic responses produced by selegiline, there is evidence for facilitation of peripheral sympathetic neurotransmission, since selegiline enhanced contractility in the field-stimulated guinea-pig and rat vas deferens [93,94]. The fact that selegiline was particularly effective in reversing the effects of clonidine in rat vas deferens may be indicative of an α-presynaptic receptor inhibitory property, but facilitation of contractions could be a manifestation of the amine-releasing property of selegiline. This effect, however, was seen at high concentration and it is not known whether a similar action occurs *in vivo*.

Other pharmacological effects of selegiline which have been observed at high concentrations of the drug include antagonism of dopamine receptors (blockade of apomorphine-induced motility at 12.5 mg/kg selegiline [74]) and inhibition of acetylcholine release in isolated striatal slices from rat brain (observed at 2.2×10^{-4} M) [92].

An effect of selegiline in the whole animal which was found at low dose (0.25 mg/kg daily for 14 days) was increased turnover rate of dopamine in the

striatum [99]. This finding is the most significant evidence in the claim that selegiline increases physiological dopamine release.

According to a model described by Knoll [99,100], brain DA which reaches its target cell after diffusing over a relatively large distance and not by intimate synaptic contact, is metabolized mainly by MAO B. MAO type B is located mainly in glial cells, the number of which, and also the brain content of MAO B, increase with age [101]. Inhibition of MAO B by selegiline is suggested to lead to increased dopaminergic activity as a result of reduced metabolism of DA by MAO B located in glial cells. On the other hand, other workers have produced evidence that DA is mainly a MAO type A substrate in rat striatum [42]. One of the actions attributed to the action of selegiline in enhancing dopaminergic activity in the brain was increased copulatory activity in sexually sluggish, aged male rats [100]. This effect was seen up to 5 days after a single dose (0.25 mg/kg) of selegiline, which tends to implicate MAO B inhibition (irreversible) in producing the effect. Other potent selective MAO B inhibitors, however, (AGN 1133, U1424, pargyline) were much less effective than selegiline in this test. The particular effectiveness of selegiline in enhancing sexual behaviour was attributed partly to its specificity for MAO type B (since 5-HT is inhibitory on sexual activity in the rat) and partly to its amphetamine-like effect. It may be that selegiline possesses a sensitive, prolonged dopamine-releasing action in the brain, even though the ancilliary actions of selegiline so far examined, and described above, have only been detected at high concentrations. It would be of interest to examine the effects of (−)-amphetamine and other MAO inhibitors in sexually sluggish rats.

CLINICAL USE OF MAO B INHIBITORS AS ADJUNCTS IN LEVODOPA THERAPY OF PARKINSON'S DISEASE

MODERN DRUG TREATMENT OF PARKINSON'S DISEASE

The fundamental works of Carlsson [102], Ehringer and Hornykiewicz [103], Birkmayer and Hornykiewicz [104], Barbeau, Murphy and Sourkes [105] and Cotzias, Van Woert and Schiffer [106] led to the DA theory of Parkinson pathogenesis and have created a firm basis for the modern therapy of PD.

Two decades after levodopa was recognized as a drug, which began a new era in the treatment of Parkinsonism, this agent still remains the drug of choice in the management of the disease.

The addition of a peripherally acting inhibitor of aromatic-amino-acid

decarboxylase (AADC) to levodopa, which protects the levodopa from being degraded in the intestine and other extracerebral tissues, has shown many therapeutic advantages. Such a combined treatment to date is well established and well documented (for review, see Ref. 107). Although levodopa is considered to be the most effective agent in the therapy of Parkinson's disease (alone or in combination with AADC inhibitors), many patients benefit from an individual combination therapy consisting of levodopa and different adjunctive antiparkinsonian medications, e.g., anticholinergics or amantadine derivatives, to achieve an optimal clinical effect. The mode of action of these drugs still remains unclear.

The treatment of Parkinsonism with anticholinergic drugs arose from an empirical or accidental chance observation. This treatment was introduced about 120 years ago by the use of extracts or pure alkaloids from the solanaceous plants, *Atropa belladonna* and *Hyoscyamus niger*. The use of these drugs showed mainly a beneficial effect on rigidity, less effect on tremor, and almost no effect on akinesia. In the last 25 years, these belladonna alkaloids have been replaced by various synthetic compounds with chemical structures apparently not resembling those of the classical antimuscarinic alkaloids [108]. Acetylcholine certainly acts as a chemical mediator in the brain and particularly in the basal ganglia [109]. Yet, it is not known whether the antagonistic effect of atropine or atropine-like substances in the CNS are due to direct actions on neurons or if they derive from the antagonism of a central synaptic transmitter [110]. Furthermore, atropine depresses not only cholinergic but also other stimuli, indicating that the drug has central actions other than blocking cholinergic synapses [111].

The antiparkinsonian effect of amantadine was also discovered accidentally by Schwab, England, Poskauzer and Young [112]. This antiviral agent [112a] has been found to be less potent than levodopa, but quite beneficial as an adjunctive drug to levodopa therapy in those patients who are only slightly disabled or in patients who do not tolerate levodopa [113,114]. The efficacy of amantadine tends to diminish with time [115]. The mechanism of action of amantadine is not clear. There is evidence to suggest that amantadine releases DA from neuronal storage sites [116] and inhibits the neuronal uptake of DA [117]. By this mode, it increases the availability of DA for neurotransmission.

In contrast to the previous therapeutic approaches, the introduction of levodopa and its therapeutic consequences are based on strong scientific concepts. The main evidence which led to these new concepts can be summarized as follows:

(1) The discovery that DA occurs in the animal and human brain [118–120].

(2) About 80% of brain DA occurs in the basal ganglia complex, the major portion in the striatum [102,121,122].
(3) The distribution of homovanillic acid (HVA), the main metabolite of DA, is more or less parallel to that of DA [121].
(4) DA and HVA concentration in striatum of Parkinsonian brain was found to be markedly (one-tenth of normal) reduced [123]. A marked reduction of DA was also found in different limbic cortical regions [124].
(5) Reduction of the activity of the DA-synthesizing enzymes, tyrosine hydroxylase and dopa decarboxylase, was found in the basal ganglia and substantia nigra in Parkinsonian brain [125].
(6) The degree of DA deficiency correlates with the loss of melanin-containing neurons in the substantia nigra, the characteristic sign of Parkinson's disease.
(7) The brain of Parkinsonian patients receiving chronic levodopa therapy had higher striatal DA and HVA than untreated patients, while good responders to levodopa accumulated more DA in striatum than poor responders [125].

All these findings justified the conclusion that the Parkinson's disease, independent of its etiology, is a striatal and limbic system-DA deficiency syndrome and the treatment of this illness with levodopa is a logical consequence of DA deficiency [126,127].

USE OF SELEGILINE IN TREATMENT OF PARKINSON'S DISEASE

The optimistic view [128] that the therapeutic effect of levodopa does not wear off in the course of time and is capable of maintaining control of the symptoms of PD has not stood the test of time. Although it is possible with an optimal medication to continue the improvement for several years [129], it became evident in clinical observations that with continued use of levodopa, more patients reported reduced efficacy with levodopa treatment, as could also be confirmed objectively [2,130,131]. Loss of efficacy is the one major problem in chronic levodopa therapy. The other serious problem in its long-term use is the periodic re-emergence of various forms of akinesia followed by periods of improved mobility, often associated with hyperkinesia. This is the so-called 'on-off' effect. Several clinical conditions are subsumed under this terminology [130,132–136]. Oscillations of motor perfomance during the day and so-called 'paradoxical' akinesia were known before the era of levodopa. These motor disturbances, however, are an integral part of the disease and cannot truly be considered as paradoxical [130]. The 'on-off' manifestations, as seen in patients who receive levodopa for a prolonged period, are more accentuated and occur with increased frequency during the day. They are usually connected with abnormal involuntary movement during the 'on' period, are related to the

levodopa dosage and are alleviated or disappear after suspending levodopa, with subsequent re-emergence of Parkinsonian symptoms. Thus, excessive levodopa administration must, in some way, be involved in the mechanism of both the dyskinesia and the temporary non-responsiveness of the receptor.

Our experience shows that there are no pure forms of 'on-off', except perhaps for the end-of-dose type, which appears in a more distinct form after reduction of the daily dose of levodopa. Moreover, the different kinds of oscillation are not constant in the same patient and usually do not appear at regular periods. They change in extent and intensity, tend to change spontaneously and are influenced by the various therapeutical approaches [136]. These circumstances make a quantitative evaluation of the conditions extremely difficult, and may be the main reason for the different assessment values stated by various authors.

A number of hypotheses have been advanced to explain the pathomechanism of the 'on-off' phenomenon [133,134,137–140]. These may be summarized as follows:

(1) A prolonged lack of DA at the striatal receptor leads to the likely development of supersensitivity in response to the neurotransmitter involved [141]; intracellularly, this may involve the induction of the striatal dopamine-sensitive adenylcyclase [142].

(2) The excessive levodopa supply is responsible for the adventitious movements, but obviously also makes the DA-receptor transiently desensitized [4] possibly through a hyperpolarization block [143]. Such effects may be caused by various dopa or DA metabolites [137,144,145], gut flora metabolites [145] or various amino acids [138] competing for the receptor. Discontinuation of the medication or its temporary reduction reverses the reactivity of the receptor.

(3) The refractoriness of the DA receptor may not be complete, since it is possible to break it by means of DA agonist [6,131,146] or selegiline [136,147], but not by levodopa. The demonstration that DA receptor agonist can reverse the motor activity in Parkinsonian patients indicates that 'off'-periods are related to fluctuations in DA availability.

There is some correlation between the plasma levodopa concentration and the 'on-off' fluctuation, i.e., 'off'-periods correlate with low plasma level and 'on'-periods with dyskinesia are seen at higher plasma levodopa levels [148,149]. Such fluctuations can be reduced by providing a relatively constant supply of levodopa. One of the clinical measures for controlling the hyperkinesia and the 'on-off' effect at present is the reduction and division of the daily levodopa doses into small, frequent doses.

The incidence of the 'on-off' phenomenon is estimated to be about 30–40% or more of levodopa-treated patients [2,132,137,150].

It is generally accepted that the disease progresses, despite continuous treatment with levodopa [2,90,107,151–153]. There is evidence indicating that the degenerative process includes both the nigro striatal DA neurons and the striatal DA receptor [126,153]. The continuous excessive supply of exogenous levodopa may account for the alterations in the properties of the central nervous system DA receptor, and is thought to be involved in the mechanism of the 'on-off' effect [4,90,130,132,154]. Hornykiewicz [126] suggested that the chronic levodopa substitution therapy abolishes the supersensitivity of Parkinsonian striatum to DA, which is a functionally important compensatory mechanism.

The application of various AADC inhibitors [107], the imposition of dietary protein restrictions [137,138], or even administration of sustained release levodopa preparations [155] can be successful in the treatment of many cases of Parkinsonism; however, they can be useful only to a limited extent in preventing the unpredictable changes in disability of the patients. All these therapeutic approaches make more levodopa available to the CNS, but they may not lead to a sustained, balanced stimulation of the DA receptor by the dopamine formed. Birkmayer [2] suggested that the main cause of the unsatisfactory response in a long-term levodopa treatment and particularly of the 'on-off' effect is a lack of DA storage and its rapid degradation by MAO.

Some theoretical considerations for the possible explanation of the 'on-off' episodes were either that uptake of levodopa by degenerated dopaminergic neurons is diminished or that the DA produced is rapidly metabolized by the enzymes MAO and COMT before or after release. This may be the reason why increasing oral levodopa doses in such cases is not effective. If 'on-off' episodes are due to lack of adequate supply of DA, it should be possible to increase its availability by the introduction of a 'safe' MAO inhibitor.

A selective inhibitor for the deamination of DA formed in the brain of Parkinsonian patients after treatment with levodopa has been regarded as desirable for many years. The first trials to potentiate and prolong the therapeutic response to levodopa with various inhibitors of MAO were made by Birkmayer and Mentasti [156]. Various available MAO inhibitors were tried together with levodopa for their clinical effect. It was shown that MAO inhibitors were able to potentiate and prolong in various degrees the kinetic effect of levodopa. This combined treatment was discontinued because of the serious side-effects. These included a toxic delirium with confusion, delusions, hallucinations and anxiety [2]. Furthermore, there was increased incidence of severe hypertensive crisis, the main side-effect of MAO inhibitors. Tranylcypromine, in combination with levodopa and peripheral AADC inhibitor, has similar effects [157-159]. The rationale implicit in this therapy is that the

therapeutic effectiveness of MAO inhibitors in Parkinson patients relies on a localized accumulation of DA at a specific site in the brain. The discovery of multiple forms of MAO with different substrate specificities and inhibitor sensitivities (see above) led Youdim, Collins and Sandler to the suggestion that syntheses of specific MAO inhibitors without the 'cheese-effect' tailored to an individual enzyme form at a particular site should be within our grasp [160].

The use of selegiline in combination with levodopa was a logical conclusion of studies showing that human brain MAO was more susceptible to inhibition by selegiline (MAO B) as compared with clorgyline (MAO A) [18], and that selegiline selectively increased brain DA levels in rats [52]. The report that selegiline does not cause a 'cheese-effect', i.e., potentiation of sympathomimetic action of tyramine [75], prompted the use of selegiline in combination with levodopa in Parkinsonian patients [3,161].

The first clinical study with selegiline as a possible therapeutic agent was based on the premise (Riederer and Youdim, 1974, unpublished data) that therapy with MAO inhibitors would be efficacious if:

(a) it were effective almost immediately after intravenous injection;
(b) no considerable side-effects ('cheese-effect') were to appear after therapeutic dosage with levodopa; and
(c) brain rather than peripheral MAO activity were specifically inhibited.

In the first clinical trial [3], selegiline was administered to 44 Parkinson patients receiving levodopa and AADC inhibitor, who had 'on-off' episodes. Selegiline (10 mg) together with levodopa (25 mg) administered i.v. during the 'off' phase resulted in a dramatic improvement: 30 min after the injection, the hitherto immobile patients were able to walk, and the perfomance of all their movements was improved. When such a combination was given intramuscularly, the onset of kinetic effect occurred later but lasted longer (1–4 days).

In an open study, selegiline in a dose of 5 mg was administered daily with the usual dosage of benserazide (Madopar) and levodopa (200 mg levodopa plus 50 mg of peripheral AADC inhibitor) three times daily. In these patients, the average disability score of 64 was reduced to 37 (a 57% improvement). The overcoming of 'off' periods was significantly improved in almost 50% of the cases. Side-effects were observed in only five out of 44 cases, including confusion (three cases) and spastic symptoms combined with abnormal movement in two cases. Hyperkinesia, which was observed in six cases, is interpreted as a potentiate of the levodopa effect rather than as a proper selegiline action.

A number of further clinical studies have confirmed the beneficial influence of selegiline on both akinesia and on the 'on-off' effect. In a double-blind, crossover trial in 86 patients [162], of whom 75 were receiving maximum

tolerated doses of levodopa with carbidopa and then who were previously untreated, selegiline, 10 mg daily, prolonged the benefits of levodopa and was helpful in 39 out of the 75 cases, who had experienced episodes of end-of-dose akinesia – or wearing-off-effect – and only one of the ten patients with 'on-off' (the 'yo-yo' type of oscillations) improved. A daily dose of 10 mg selegiline made a mean dosage reduction of 200 mg of levodopa possible. On this dosage, only four patients showed an amphetamine response, but this was more frequent when 40 mg selegiline was administered daily. Four of 85 patients derived sustained increase in energy, alertness and mental concentration. In ten patients, when the daily dose of selegiline was increased to 20 mg, no further antiparkinsonian effect was seen, but there was an elevation of mood accompanied by pupillary dilatation and sweating. Side-effects, in order of frequency, were nausea, dry mouth, confusion with occasional visual hallucinations, dizziness and headache; insomnia and pressure of speech were seen occassionally when the dose of selegiline exceeded 10 mg daily. Dyskinesia was frequently seen when selegiline was added to levodopa and carbidopa, but cleared as the dose of levodopa was reduced. A balanced crossover study in six healthy young male adults showed that selegiline was associated with a significant increase in the frequency of periods of wakefulness and stage 2 sleep, and a significant decrease in REM sleep and sleep stages 3 and 4.

Contrary to these findings, Lavie, Wajsbort and Youdim found that selegiline does not cause insomnia in Parkinsonian patients [163]. Selegiline, 10 mg daily, was administered to ten Parkinsonian patients treated with levodopa. Whole-night electroencephalographic, electro-oculographic and electromyographic recordings were made on each patient for evaluation of sleep structure before treatment and 14 days after treatment had begun. A dose of 10 mg selegiline significantly shortened sleep latency without affecting any of the other sleep parameters. Although there were fewer episodes of REM with selegiline treatment, REM was lower by only 6%.

It seemed that sleep improved somewhat with selegiline. Besides the decrease of sleep latency, sleep efficiency index and percents of sleep stages 2 and 3–4 increased, while there was a decrease in the percent wake-plus-movement time and in percent stage 1. In this study, 10 mg daily dose of selegiline was sufficient to inhibit platelet MAO activity almost by 100% (93 ± 5) in all ten patients.

Rinne, Sirotola and Sonninen [131] reported their experience in 47 patients in Parkinson's disease under chronic levodopa therapy with and without 'on-off' phenomena. Twenty-three out of 34 (68%) during 1–3 months treatment with 5–10 mg of selegiline showed a significant reduction in 'on-off' changes. The degree of improvement was defined as minimal (30%) or

moderate (38%). All patterns of 'on-off' disabilities gained benefit. Peak-dose dyskinesias were aggravated in about half of the patients. Dystonic disturbances showed variable responses, but muscle cramps improved in four patients. A significant further improvement in Parkinsonian disability developed during treatment with levodopa and selegiline. All the main Parkinsonian symptoms improved, but the improvement of tremors seemed somewhat better than that of rigidity and hypokinesia. Contrary to the assertion of Birkmayer [2] that selegiline has only a potentiating effect on levodopa when combined with a peripheral AADC inhibitor, i.e., selegiline plus levodopa (orally) alone is not as effective. Linne, Sirotola and Sonninen [131] found no difference in the response to selegiline between patients treated with levodopa alone and those treated with levodopa combined with AADC inhibitor.

In patients without 'on-off' effect, only six out of 13 showed an improvement in Parkinsonian disability, and the mean improvement was less marked than in patients suffering from 'on-off' phenomena. Side-effects occurred somewhat more frequently during combined treatment with levodopa and selegiline than with levodopa alone.

The authors concurred that selegiline is a valuable adjuvant, at least for certain patients, especially those with 'on-off' complications of chronic levodopa therapy.

In the study by Yahr [90], 35 patients whose Parkinsonism had been treated for an average of 7.5 years were studied. Twenty-nine of them suffered from the 'on-off' effect. Selegiline in a dose of 10 mg/day added to the previous levodopa-carbidopa or -benserazide combination ameliorated the 'on-off' oscillations to a considerable extent. An improvement of Parkinsonian state, but concomitantly an increase in abnormal involuntary movements, was observed. In general, responses were evident within 3 days of initiating the combined drug programme. The addition of selegiline in six patients who, over time, lost more than 50% of their responsiveness to levodopa, resulted in an improvement of 25% of the Parkinsonian state in half of them. No serious side-effects were observed. The levodopa-sparing effect of selegiline administration was on average 10%. These results have largely been confirmed by other workers [164,165].

In our clinical and biochemical investigation [136], the effect of selegiline in combination with levodopa plus carbidopa (Sinemet) was studied in 22 Parkinsonian patients with various forms of 'on-off' phenomena. All patients were previously chronically treated by the L-carbidopa combination for many years. Carbidopa (MK 486) is a peripheral decarboxylase inhibitor. All but three of the 22 patients showed some improvement of the Parkinsonian symptoms following addition of selegiline. A significant improvement of the

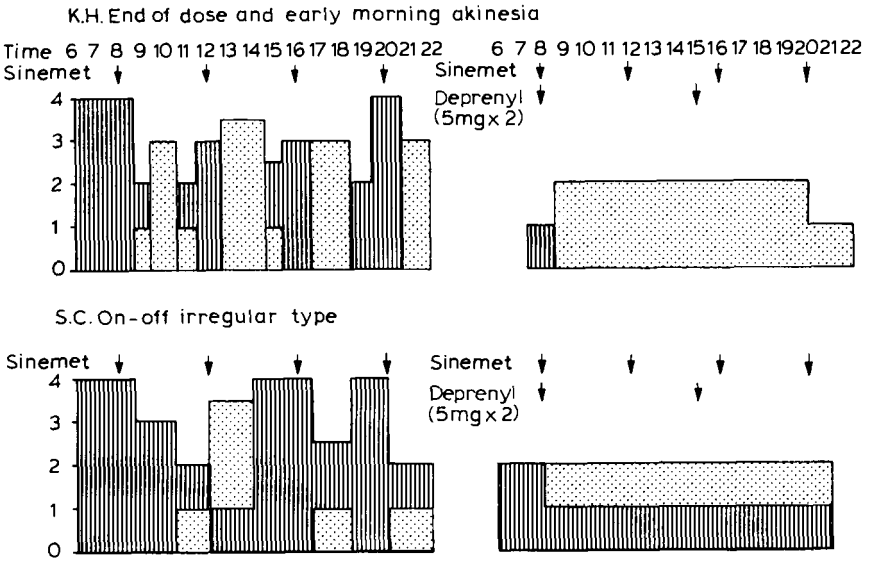

Figure 4.1. Comparison of clinical response in two Parkinsonian patients with 'on-off' phenomenon treated with either carbidopa plus levodopa (Sinemet) alone or in combination with selegiline ((−)-deprenyl). S.C.63, female, Parkinson's disease-levodopa treatment, 9 years. Sinemet AVG daily dose 75/750. K.H.54, male, Parkinson's disease 10 years. Levodopa treatment 10 years. Sinemet AVG daily dose 75/750. Lined box, akinesia and dotted box, hyperkinesia. Patient perfomance graded as excellent (1), good (2), satisfactory (3) or bad (4). Taken from Ref. 136.

'on-off' phenomenon occurred in 18 cases, with a disappearence in nine of them (*Figure 4.1*). More than half the group studied showed signs of improvement which were defined as 'good' or 'very good' and only one of three patients in the 'poor' improvement group showed a rapid deterioration of his condition in the follow-up observation. He was thought to be suffering from the malignant type of PD [148]. Selegiline significantly influenced all types of the 'on-off' effect, as categorized by us (nocturnal akinesia, early morning akinesia, end-of-dose akinesia, non-dose-related akinesia, or mixed type). In 11 out of the 16 patients with abnormal involuntary movements prior to selegiline treatment, augmentation of the dyskinesia was observed.

The side-effects which were observed or complained of during the selegiline treatment are not qualitatively or quantitatively different from those reported for levodopa. The 10 mg daily dose of selegiline was sufficient to inhibit platelet MAO-activity completely and the inhibition was maintained while patients received selegiline. There were no changes in the platelet aggregation responses to 5-HT or ADP during the treatment with selegiline.

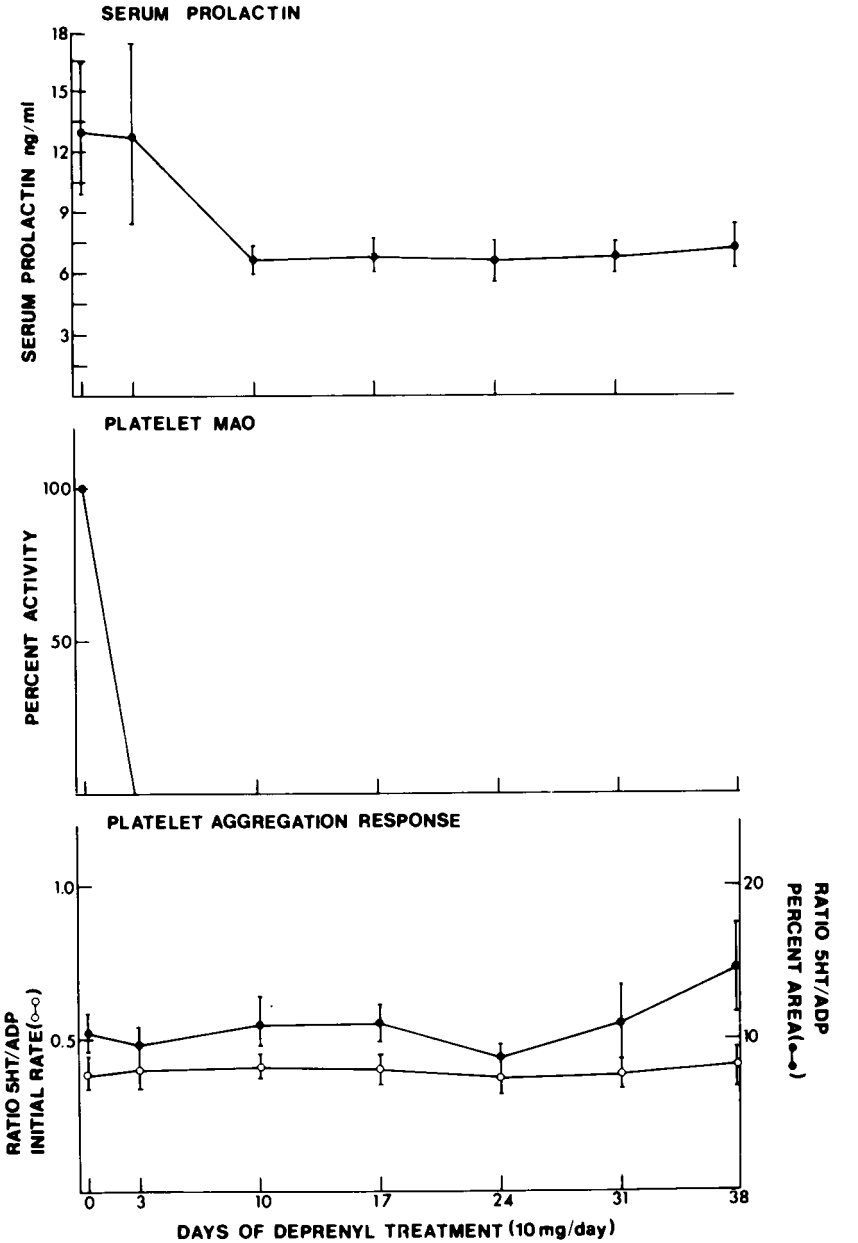

Figure 4.2. Serum prolactin, platelet monoamine oxidase (MAO), and platelet function in 25 Parkinsonian patients treated with carbidopa plus levodopa (Sinemet) and selegiline ((–)-deprenyl). Taken from Ref. 136.

The serum prolactin concentration decreased slightly but not significantly during selegiline administration (*Figure 4.2*). The mean daily levodopa dosage while selegiline was administered could be reduced by about one-third. The explanation for the required reduction of levodopa seems consistent with the fact that 10 mg selegiline is sufficient to inhibit almost completely MAO type B [166], for which DA is a substrate. Thus, DA formed from exogenous supply of levodopa cannot be deaminated and accumulates at the intact DA nerve endings. In circumstances where MAO is intact, larger amounts of levodopa are required, since the DA formed can be deaminated either intraneuronally of after release.

The results of a long-term study with selegiline were recently reported by Birkmayer, Knoll, Riederer and Youdim [167]. Two hundred and eighty-five patients with PD receiving selegiline 10 mg as a single dose or 2 × 5 mg per day in addition to chronic levodopa therapy (Madopar or Sinemet) were compared with 325 patients who received the above levodopa therapy alone. The duration of selegiline administration varied from 2 to 8 years.

Fourteen patients with and 96 without selegiline treatment died during the course of the disease. The combined therapy with selegiline led to a significant prolongation of the evolution of the disease. The average improvement in 323 patients on conventional antiparkinsonian therapy was 27%, while in 285 patients with additional selegiline therapy the degree of improvement was 37%.

The occurrence of side-effects of adverse reaction was similar in patients treated by the levodopa therapy and in those treated by the combined therapy with selegiline. In only one double-blind study with selegiline on 11 patients were negative results demonstrated [168]. Two patients did not complete the study: one because of psychotic side-effects and another who refused to continue because of apparent worsening of 'on-off' swings after insisting on an increase in Sinemet dosage.

The last case reflects, in our opinion, the view of Duvoisin [169] that high doses of levodopa are one of the main reasons responsible for the occurrence of the 'on-off' effect, and have, therefore, to be avoided. In the nine cases who completed the trial, the authors were unable to demonstrate any substantial benefit of selegiline when added to optimal chronic treatment with carbidopa-levodopa (Sinemet). Furthermore, they concluded that any advantages deriving from the use of selegiline in Parkinsonism are probably due to its mood-elevating effect [168]. However, this negative report is isolated among several trials which leave no doubt about the action of selegiline in potentiating the antiparkinsonian effect of levodopa and especially its positive influence on the 'on-off' phenomenon.

CONCLUSION

Selegiline, a potent MAO inhibitor, showed in almost all [3,90, 127,131,135,136,162,164,165] but one [168] clinical studies, a definitive potentiating effect in the therapeutic response of levodopa alone or in combination with a peripheral AADC inhibitor in PD. From the clinical point of view, there is a distinct influence of selegiline on different aspects of Parkinsonism, which can be summarized as follows:

(a) Potentiation of levodopa effect, including abnormal involuntary movements.

(b) Prolongation of levodopa action and positive influence on the 'on-off' phenomena.

(c) The ability to improve the function of the dopaminergic system even in advanced cases, where, according to all predictions, a serious degeneration of the DA neurons exists.

(d) It does not cause serious side-effects different from those seen during the conventional antiparkinsonian therapy.

(e) Its beneficial effect in PD is optimal at the dose of 5–10 mg/day.

(f) It probably acts as an 'energizer' in geriatric patients.

APPENDIX

MEASUREMENT OF MAO TYPE B ACTIVITY

This is conveniently carried out using a selective MAO type B substrate, either PEA or benzylamine. When PEA is used, the concentration should be 20 μM, since at higher concentrations this substrate is also deaminated by MAO type A [170]. Since this concentration is close to the K_m for MAO type B, care must be taken that only a small amount of substrate is oxidized in the course of the reaction. The use of benzylamine is preferred by many workers, since this substrate is not inhibitory on the reaction, as has been reported for PEA [171].

The ^{14}C-labelled substrates are incubated with a mitochondrial preparation of the tissue, or with a crude whole homogenate, in a Tris buffer under conditions permitting ready access of oxygen to the medium, and oxidized products separated from amine substrate by a simple ion-exchange resin or by solvent extraction [172].

Inhibitory potency *in vitro* is estimated by inclusion of several concentrations of the inhibitor in the medium. To determine *in vivo* effectiveness of irreversible inhibitors, tissue homogenates of suitable injected animals are incubated with

one of the above substrates. In man, MAO type B inhibition is conveniently determined by examining platelet homogenates, since in this tissue most of the MAO activity is of type B.

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5 Recent Studies on Doxorubicin and its Analogues

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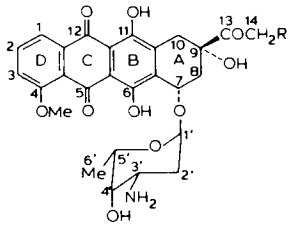
INTRODUCTION

In the 15 years which have elapsed since the isolation of doxorubicin* (1)

Parent Antibiotics



(1) R = OH doxorubicin (2) R = H daunorubicin

was first reported [1], this drug has become established as one of the most useful in the oncologist's armamentarium (for a review of its development see Ref. 2). Its indications include lymphomas (Hodgkin's and non-Hodgkin's), acute leukaemias, osteogenic and soft-tissue carcinomas, solid tumours (particularly of the breast and ovaries and to a lesser extent of the bladder and lung) and some paediatric tumours [3]. Like all antitumour drugs, it is usually used in combination therapy. Doxorubicin is one member of the anthracycline group of antibiotics (which are glycosides containing a tetrahydronaphthacenequinone aglycone) and is produced by a variant of the organism which produces daunorubicin (2) (trivial name, daunomycin), the latter drug being preferred in the treatment of acute leukaemia (particularly acute non-lymphocytic leukaemia), since it is equiactive here and lower in cost. Because of the pre-eminence of doxorubicin, daunorubicin (which was in fact its forerunner) has perhaps received less attention than it merits and a rigorous comparison of the two drugs in treatment of solid tumours is timely [4] (for a review of the development of daunorubicin see Ref. 5). As a result of their clinical importance, the anthracyclines have been the subject of many books, conferences and review articles (for example, Refs. 2,3,6–25).

Despite its good antitumour activity, doxorubicin is far from an ideal drug as it has both pharmaceutical and toxicological limitations. Although stable in the solid state, it is photolabile via a free-radical process which leads to aglycones and aglycone polymers [26–28] and so degrades in dilute solution, the rate increasing with increasing dilution and with increasing light intensity

*Doxorubicin is the international non-proprietary name; the compound is also known under the trivial name adriamycin, which is also its trade name.

[26] (it is, however, stable under normal lighting conditions at the concentration at which it is injected into fast-running i.v. lines). It adsorbs onto glass surfaces [29], onto metal (e.g., syringe needles) [29,30], chelates divalent metal ion salts (including bone) [31,32] and aggregates in solution [33,34]. It interacts with heparin, so reducing the anticoagulant effect of the latter [35]. It is not orally active, so is given by i.v. infusion [3]; the risk of extravasation must, however, be avoided, as this leads to severe local tissue necrosis which can go as deep as tendon and bone [36]. It is irritant to mucous membranes [3]. Acute effects which are not dose-limiting include nausea and vomiting [3], effects due to histamine release from mast cells [37] ranging from hypotension to local, regional or generalized urticaria to anaphylactic shock [37,38], and acute cardiac effects [3]. Longer-term toxic effects include stomatitis (which may necessitate dose reduction), recall of radiation toxicity, alopecia (in all patients) [3], nephrotoxicity (rare), neurotoxicity (very rare), reduction of gonadal function, hyperpigmentation [39], mutagenic and teratogenic effects [40,41], immunosuppression, bone marrow suppression [18] and chronic cardiotoxicity (which is unrelated to the acute cardiotoxicity) [3]. This latter chronic cardiotoxicity is the major dose-limiting effect of doxorubicin, and occurs as a fatal cardiomyopathy, the incidence increasing with the cumulative dose. As the incidence is up to 7% at 550 mg m^{-2}, the total dose is usually limited to this figure and the drug must only be used with caution above this total cumulative dose. Predisposing factors include advanced age, previous history of cardiovascular disorders, therapy with mitomycin, dactinomycin, cyclophosphamide or mithramycin [42].

There are three approaches to ameliorating the disadvantages of this drug: firstly improved usage of the drug, secondly development of improved analogues, and thirdly improved targetting to the tumour cells. Considering usage of doxorubicin itself, improved methods are needed to determine if a particular patient will respond to the drug. Methods for reliable *in vitro* testing of xenografts of human tumours (in nude mice) for colony forming units after exposure to doxorubicin or for perturbation of cell cycling by the drug are now being developed [43–46]: the aim is therefore to identify those patients with tumours sensitive to doxorubicin. A further aim is to then maximize therapeutic effectiveness in these patients whilst minimizing toxicity, and improvements in scheduling [47–49] have indeed been shown to improve the therapeutic index. More pharmacokinetic studies are needed, however, to identify those parameters that relate to response; for example one study has shown that with breast cancer it is the clearance pattern in the initial distribution phase which correlates with therapeutic effectiveness [50]. Correlation of all such data along with known pharmacokinetic data (such as Refs. 51–56) and introduction of

routine therapeutic drug monitoring will be necessary to optimize usage of this drug fully. Following this approach will not only improve antitumour effectiveness, but should also give minimization of potential cardiomyopathy; currently, biopsy and histological examination is the most effective monitor of impending chronic cardiotoxicity [57], and a reliable non-invasive method would naturally be preferable. Considering analogues and targeting, well over 500 analogues or derivatives have been prepared (synthetically and, mainly, semisynthetically) or isolated from micro-organisms and, as described later, several are in clinical trial. We can look forward therefore to a continuing improvement in the clinical application of anthracyclines.

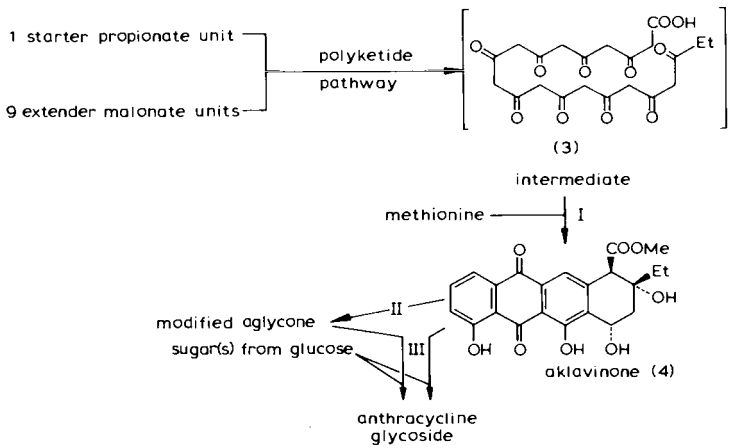
This review will cover the period since the last review on doxorubicin in this series [11] and will focus on three areas;

(a) synthetic routes to doxorubicin and other anthracyclines;
(b) biochemical effects of doxorubicin; and
(c) analogues (and derivatives) of doxorubicin.

In order to discuss these areas, we must first, however, appreciate the structural relationships within the anthracycline group of compounds.

INTERRELATIONSHIPS BETWEEN ANTHRACYCLINES

The general pathway for anthracycline biosynthesis is shown in *Scheme 5.1* [58–63].

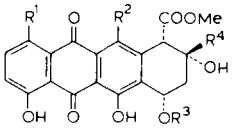


Scheme 5.1.

(I) Usually the oxygenation on that carbon atom in the 'intermediate' (3) which is destined to become C-2 of the anthracyclines, is lost; also the carboxyl group is usually esterified to the methyl ester (the methyl unit is from methionine [64]). Aklavinone (4) is therefore the archetypical initial product.

(II) Aklavinone (4) may be further processed, for example, by oxidation at C-1 to give ε-pyrromycinone (5), at C-11 to give ε-rhodomycinone (6), or (less commonly) at both C-1 and C-11 to give ε-isorhodomycinone (7).

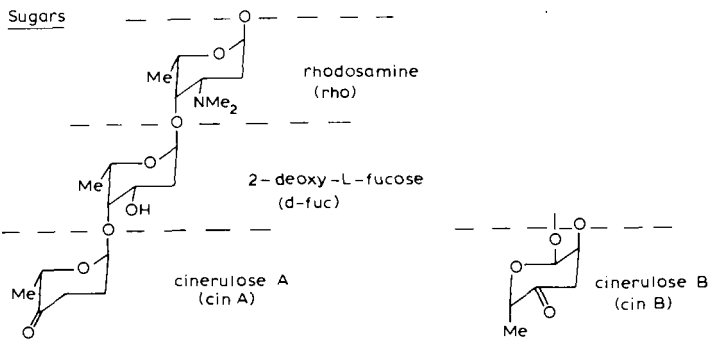
(III) The aglycones can be glycosylated sequentially [65] to give anthracyclines with mono-, di- or oligosaccharide chains, e.g., ε-pyrromycinone can give pyrromycin (8), musettamycin (9) and marcellomycin (10) [66]. Generally

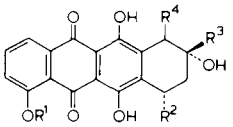


Compounds with a 10-methoxycarbonyl group

	R^1	R^2	R^3	R^4	
(4)	H	H	H	Et	alkavinone
(5)	OH	H	H	Et	ε-pyrromycinone
(6)	H	OH	H	Et	ε-rhodomycinone
(7)	OH	OH	H	Et	ε-isorhodomycinone
(8)	OH	H	rho	Et	pyrromycin
(9)	OH	H	rho-d-fuc	Et	musettamycin
(10)	OH	H	rho-d-fuc-d-fuc	Et	marcellomycin
(14)	H	H	rho-d-fuc-cin A	Et	aclacinomycin A
(15)	H	H	rho-d-fuc-cin B	Et	aclacinomycin B
(21)	OH	H	rho-d-fuc-cin A	Me	1-hydroxyaurramycin A
(22)	OH	H	rho-d-fuc-cin A	CH_2Ac	1-hydroxysulfurmycin A

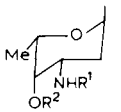
d = 2-deoxy





Compounds without a 10-methoxycarbonyl group

	R^1	R^2	R^3	R^4	
(11)	H	OH	Ac	H	carminomycinone
(12)	H	O-daunos.[a]	Ac	H	carminomycin
(13)	Me	O-daunos.	CH(OH)Me	H	dihydrodaunomycin
(16)	Me	O-daunos. deriv. A[b]	Ac	H	baumycin A_1
(17)	H	O-daunos. deriv A[b]	Ac	H	rubeomycin A
(18)	H	O-daunos. deriv B[c]	CH(OH)Me	H	N-formyldihydrocarminomycin
(19)	H	O-rho	Et	O-rho	β-rhodomycin II
(20)	H	H	Et	O-rho-rho	roseorubicin B
(26)	H	O-rho-d-fuc-d-fuc	CH(OH)Me	H	trisarubicinol
(27)	H	H	Et	O-rho	iremycin



	R^1	R^2
a daunosamine	H	H
b daunosamine deriv A	H	CHOCHMeCH$_2$OH CH$_2$CHMeOH
c daunosamine deriv B	CHO	H

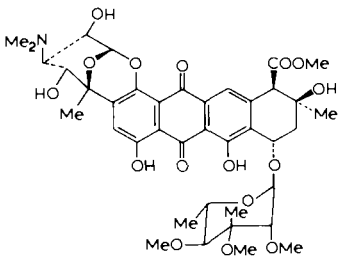
the primary sugar is an aminosugar (commonly rhodosamine, as in (8), (9) and (10)); these sugars are biogenetically derived from glucose [64].

Modification at other centres in these aglycones can occur subsequent to their formation and prior to glycosylation; for example, ε-rhodomycinone (6) can be demethoxycarbonylated, and oxidized to carminomycinone (11) which is then glycosylated to carminomycin (carubicin) (12) [61]. As a further example, ε-rhodomycinone can be demethoxycarbonylated, 4-*O*-methylated and then glycosylated with the same sugar as in carminomycin (daunosamine) to give, after side-chain modification, dihydrodaunorubicin (13). Further modification in the aglycone may occur once the glycoside has been formed; for example, the aforementioned dihydrodaunorubicin (13) may be oxidized to daunorubicin (2) [61,67], and if 14-hydroxylation additionally occurs, then doxorubicin (1) is formed [68,69]. Also modification of the sugar unit can occur once the glycoside has been formed, either by oxidoreductases, for example, conversion of the aklavinone glycoside aclacinomycin A (aclarubicin) (14) to aclacinomycin B (15) [70] or by formation of derivatives at oxygen or nitrogen centres, for example, the baumycins (e.g., baumycin A_1 (16)) [71], the

rubeomycins (e.g., rubeomycin A (17)) [72] and *N*-formyldihydrocarminomycin (18) [73]. Other known modifications include 10-demethoxycarbonyl-10-hydroxy compounds e.g., β-rhodomycin II (19) [74] and 7-deoxy-10-hydroxy compounds such as roseorubicin B (20) [75]. Hence, there is a whole range of permutations of natural structures derived from the initial product, aklavinone (4).

In the compounds discussed so far, the starter unit has always been propionate; substitution of this starter by acetate or by 2-ketobutyrate can occur giving a range of parallel products, for example, 1-hydroxyauramycin A (21) and 1-hydroxysulfurmycin A (22) [76], parallel marcellomycin (10). Other compounds with acetate as starter unit [77] include nogalamycin (23) [78], decilorubicin (24) [79] and steffimycin A (25) [80].

In addition to these natural anthracyclines, mutant organisms can be derived in the search for new anthracyclines (doxorubicin itself is in fact derived from a mutant derived by exposure of the daunorubicin-producing organism to a



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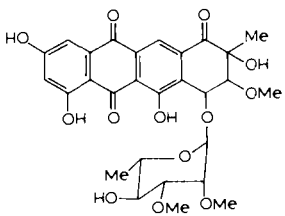

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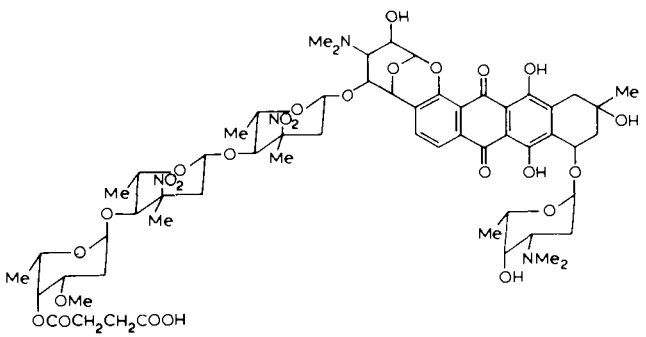
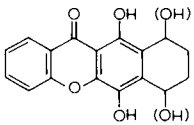


(24)

mutagenic agent). Also, blocked mutants can be used to produce novel anthracyclines [81,65]; for example, feeding of aglycones other than aklavinone (4) to a blocked mutant of the organism which produces aclacinomycin A (14) gives corresponding glycosides with the trisaccharide unit of aclacinomycin A; for example, trisarubicinol (26) is formed from carminomycinone (11) [82] (note that also the 13-keto group is reduced in this example). Further novel compounds can be produced by recombinants of *Streptomyces* spp.; for example, iremycin (27) has been derived in this way [83].

A range of anthracyclines from natural organisms and from derived organisms are therefore available; the activities of the most significant compounds will be discussed later. Most of the multitude of doxorubicin analogues have been produced from 'natural' anthracyclines by semi-synthesis. Work on total synthesis has (disappointingly) been mainly concerned with development of routes to the natural anthracyclines: only recently have attempts been made to synthesize anthracyclines containing novel aglycones, for example, (28–31) [84–87].



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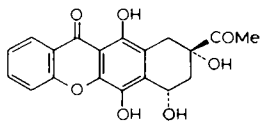

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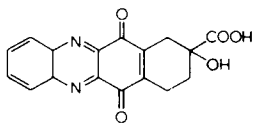

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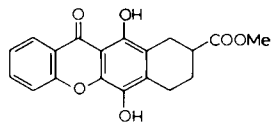


(31)

SYNTHESIS OF DOXORUBICIN AND OTHER ANTHRACYCLINES

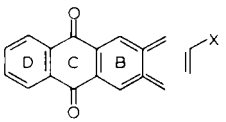
Most of the work on synthetic routes to anthracyclines has concentrated on doxorubicin (1) and the related compounds, daunorubicin (2) and carminomycin (12). The stimulus has been the low yield of doxorubicin (1) from the

fermentation process (and hence high cost) and also the challenge of synthesis of a compound of such complexity. Only recently has attention really widened to synthesis of other natural anthracyclines. Apart from one recent study, all work has started from the premise that glycosidation should be the final step; synthesis of the aglycone has generally therefore been the first consideration. The major problem in design of a route is to obtain regiochemical and stereochemical control.

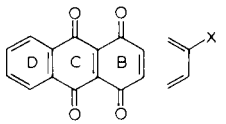
DIELS-ALDER REACTION

Retro-analysis shows that the Diels-Alder reaction could be used to construct the tetracyclic ring system from synthetically accessible precursors whilst retaining any chirality in these precursors. The possible strategies are as follows:

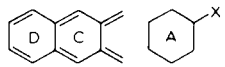
(a) DCB diene synthon:



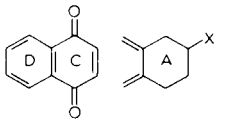
(b) DCB dienophile synthon:



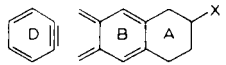
(c) DC diene synthon:



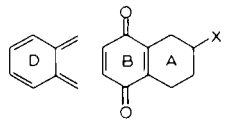
(d) DC dienophile synthon:



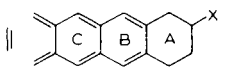
(e) BA diene synthon:



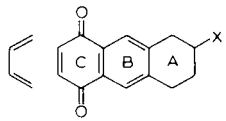
(f) BA dienophile synthon:



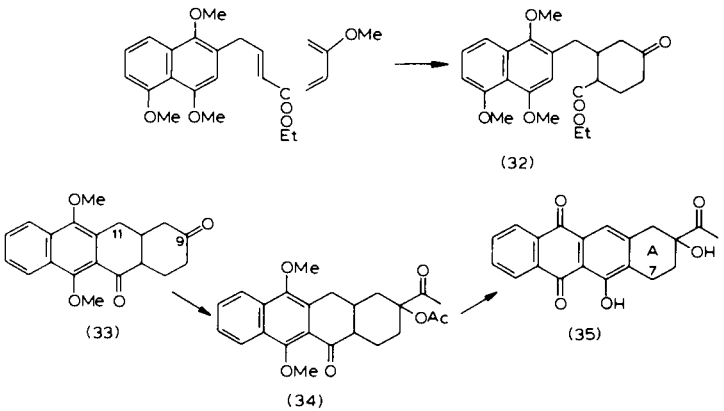
(g) CBA diene synthon:



(h) CBA dienophile synthon:



The Diels-Alder approach has been the most widely used in anthracycline synthesis and most of the above strategies have been used. Additional variations which have been studied include the route [88] (*Scheme 5.2*) designed to give 11-deoxyanthracyclines.



Scheme 5.2.

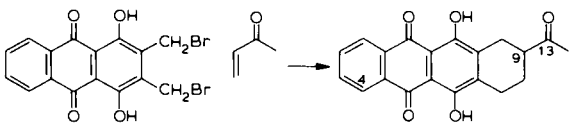
Hydrolysis and cyclization of (32) gave the tetracycle (33) which on reaction with ethynylmagnesium bromide, then $Hg(OAc)_2$-H_2S, gave (34) which was demethylated and aromatized to (35). Benzylic bromination and hydrolysis gave the 7-hydroxy derivative of (35).

The (non-stereospecific) methods used here to functionalize the A ring from a 9-keto group are the 'classical' methods of A-ring functionalization [89,90].

Each of the Diels-Alder strategies a–h (above) will now be discussed in turn.

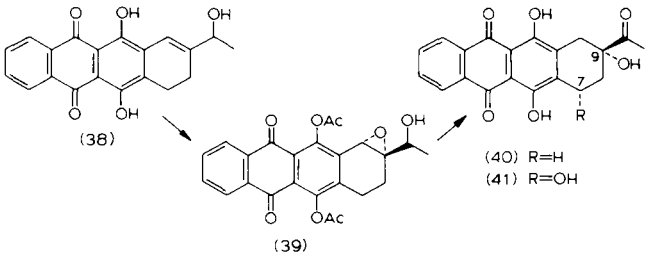
(a) Diels-Alder reaction – DCB diene synthon approach

Cava and co-workers [91–94] have used this strategy as a route to 4-demethoxydaunorubicinone, the aglycone of 4-demethoxydaunorubicin. Free-radical bromination of 1,4-dimethoxy-2,3-dimethylanthraquinone gave (36). Generation of the quinonedimethane in the presence of methyl vinyl ketone yielded (37).

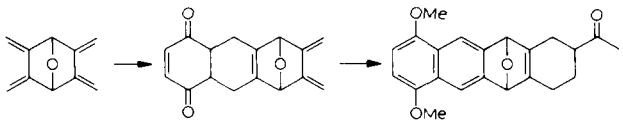


Stereospecific hydroxylation of ring A was achieved by successive bromination at C9, elimination of HBr and selective reduction at C13 to give racemic (38).

After protection of the phenolic groups, epoxidation was carried out *stereospecifically* to give (39), which was readily converted to (40); 'classical' methods could then be used to introduce the 7-hydroxy group stereospecifically *cis* to the 9-hydroxy group [95], giving 4-demethoxydaunorubicin (41).



This DCB diene synthon approach has also been exploited by Vogel and co-workers [96,97], who reacted a norbornane C-ring precursor (42) with benzoquinone to give the synthon (43) which can be aromatized, methylated, oxidized and reacted with methyl vinyl ketone to give (44).

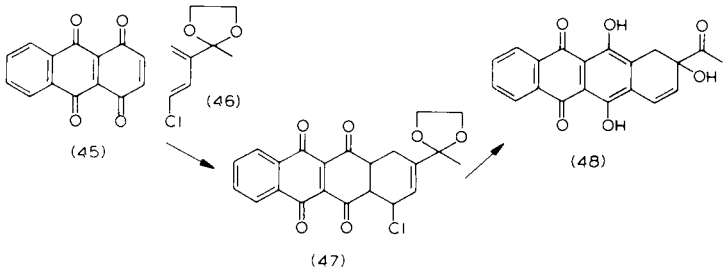


This 'double Diels-Alder' route gives access to a range of anthracyclinones (aglycones of anthracyclines) including daunorubicinone (via selective reduction of (43), the subsequent reaction with methyl vinyl ketone giving the right regiochemical isomer as the major product).

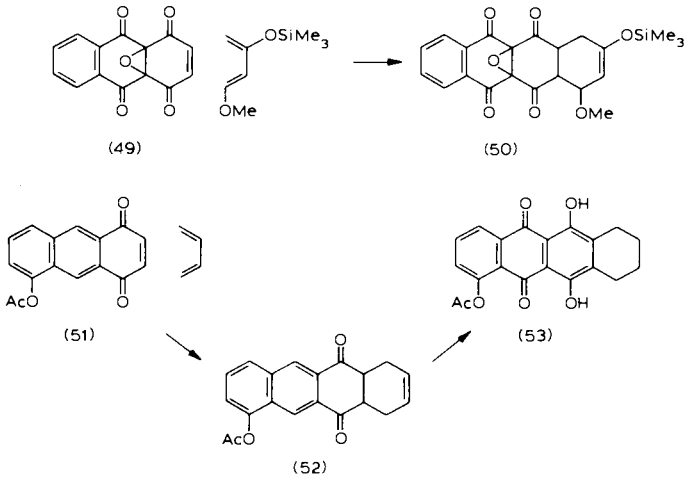
(b) Diels-Alder reaction – DCB dienophile synthon approach

Although Diels-Alder reaction of quinizarinquinone (45) (or a substituted quinizarinquinone) would appear to be the most direct route to anthracyclinones, there are two problems; firstly, internal adducts may be formed as well as or instead of the desired end adducts, and, secondly, regiochemical control is needed. Considering the first problem, internal adduct formation can be avoided by use of an appropriate diene; for example, the chlorobutadiene

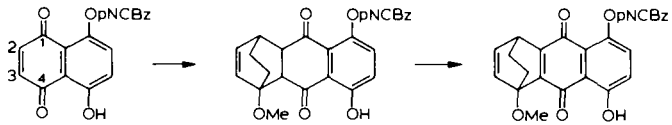
analogue (46) gives an end adduct (47) which can be converted to (48) [98], whereas the methoxy analogue gives an unwanted internal adduct.



Alternative approaches include protection of the internal double bond as an epoxide as in conversion of (49) to (50) [99–101] or reaction of the diene with an anthracene-1,4-dione, for example (51) to (52), followed by oxidation to the tetrahydronaphthacenequinone (53) [102–105].

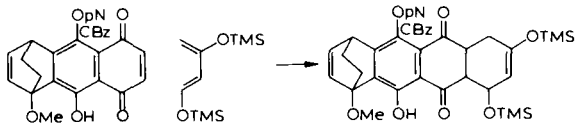


Turning to regiospecificity, the regiochemical control in the addition of dienes to quinones has been well studied [106–110]. Regiocontrol can be obtained by using a monoacylated dihydroxyquinone; for example, (54) with 2,3-dihydroanisole gives (55), which can be oxidized to (56) [111] (pNCBz denotes *p*-nitrocarbobenzoxy).



(54) (55) (56)

Hydrogen bonding to the C4 carbonyl and electron donation of the ester into the C1 carbonyl renders the C4 group the most electron-deficient group on the C2/C3 bond. Compound (56) will exist to a minor extent as the tautomer (56a) so further Diels-Alder reaction can occur to give, for example, (57) [111]; masking of the D ring is essential here, as this tautomerization and reaction will not occur if the D ring is aromatic [112].



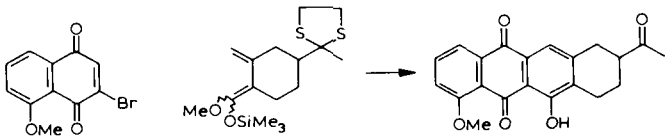
(56a) (57)

(c) Diels-Alder reaction – DCB diene synthon approach

There are no reported syntheses which use this approach.

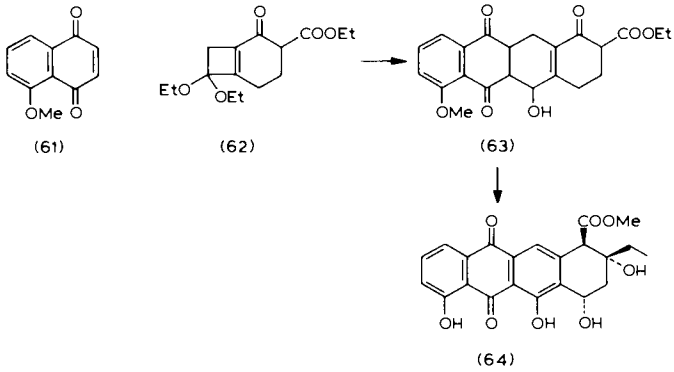
(d) Diels-Alder reaction – DCB dienophile synthon approach

Cycloaddition of suitable vinyl ketone acetals [113–115] or dienes [116] to naphthoquinones has been reported; for example, (58) with (59) gives, after acidic work-up and oxidative cleavage of the dithian group, the 11-deoxyanthracyclinone (60) [117]. Regiospecificity is assured by use of the appropriately substituted naphthoquinone. Hydroxylation [116] of (60) at C9 and C7 was carried out by 'classical' methods [118,95].



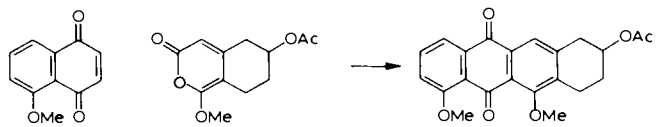
(58) (59) (60)

Generation of the diene from a cyclobutane has also been used [119,120]; for example, (61) with (62) gives (63), which was further converted to (64) [121].



(61) (62) (63) (64)

Further, pyrones have been used as the diene component, and this is found to lead to regiospecificity [122,123]; for example, (65) and (66) give (67) selectively:

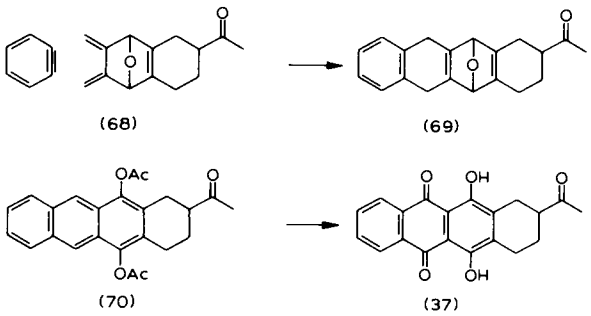


(65) (66) (67)

The advantage of this overall strategy is that a wide range of anthracyclinone substitution patterns is accessible [124].

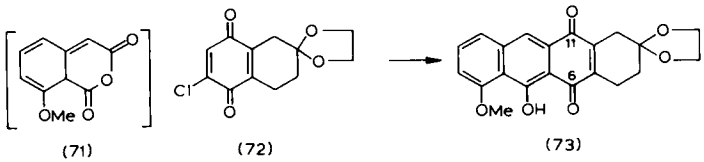
(e) Diels-Alder reaction – BA diene synthon approach

This approach is exemplified by reaction of dehydrobenzene with norbornane (cf. (a) above) (68) to give (69) [125]. This can be aromatized and further converted to (70), which can be oxidized to the quinone and deacetylated to (37).

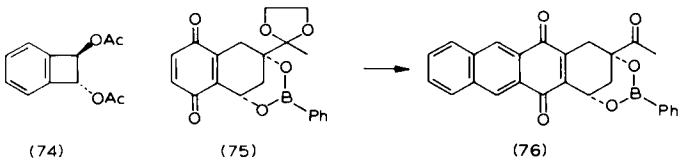


(f) Diels-Alder reaction – BA dienophile approach

This is a well researched method, since choice of a suitable tetrahydronaphthoquinone as dienophile gives ready access to the 6,11-dihydroxy-(ε-rhodomycinone and daunorubicinone) group of aglycones. As an example, equivalent to the pyrone approach mentioned under (d) above, regiospecific addition of a homophthalic anhydride, as tautomer (71), to an appropriate quinone (72) gave (73) [126].



Also, cyclobutanes and *o*-di(bromomethyl)benzene derivatives have been used as *o*-benzoquinodimethide precursors [127–130]. In all these methods, the preparation of the quinone dienophile is a prime consideration; for example, in one synthesis, full functionality was introduced into the quinone and cycloaddition of (74) to this quinone (75) gave the 4-demethoxydaunorubicin precursor (76) [131,132].

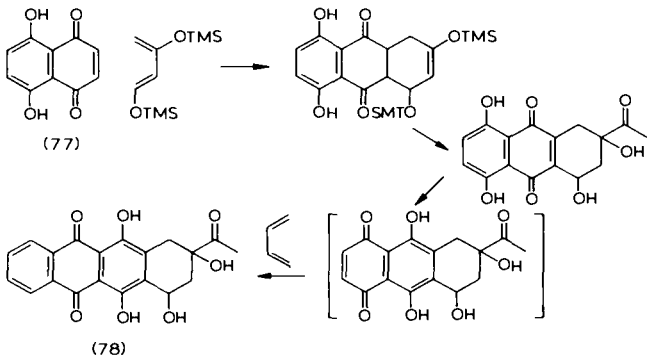


(g) Diels-Alder reaction – CBA diene synthon approach

This approach has not been used: it offers no merit.

(h) Diels-Alder reaction – CBA dienophile synthon approach

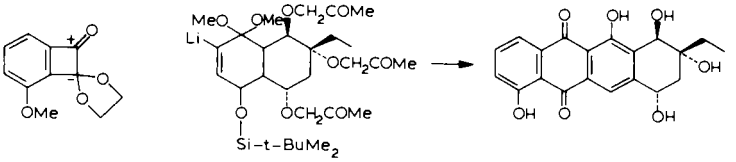
This process has been used principally as an 'end reaction' for a double Diels-Alder sequence on a dihydroxynaphthoquinone. Consequently, reaction of dihydroxynaphthoquinone (or its dimethoxy or diacetyl derivative) with a diene [133–138] or with sulpholene [139] followed by oxidation back to the quinone yields a compound which can tautomerize and undergo a further Diels-Alder addition. Functionalization can be carried out prior to this final Diels-Alder reaction, as for example, (77)→(78) [133].



In summary, we should consider the comments of Kelly in his 1979 review [140] of anthracycline synthesis '...the power of the Diels-Alder reaction for the expeditious construction of complex carbocycles has not escaped the notice of practitioners of anthracycline synthesis...'. Indeed, over half the methods covered in the review by Kametani and Fukumoto [141] used the Diels-Alder reaction for ring skeleton construction.

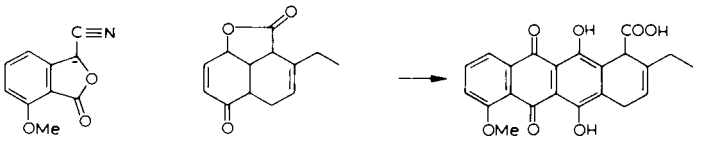
1,4-DIPOLAR STRATEGY

Some regiochemical control can be achieved as seen above. Regioselectivity can be assured in anthracycline synthesis via the '1,4-dipole strategy' in which a 1,4-dipolar compound such as (79) is reacted with a lithiated quinone derivative [142–146], for example, (80), yielding in this case (81).



(79) (80) (81)

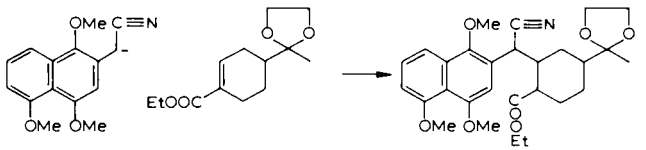
Routes with a 'reversed' polarization of reactant are perhaps even more effective, and anions of phthalides [147], of phthalidesulphones [148–151], of cyanophthalides [151,152] and of dimethylhomophthalates [153] have been used; for example, (82), formed at $-78\,^{\circ}C$ with lithium diisopropylamide in THF in presence of hexamethylphosphoramide, with enone (83) gives (84).



(82) (83) (84)

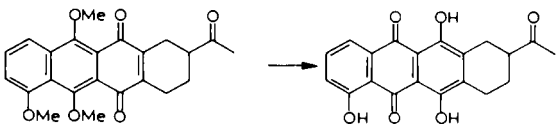
NUCLEOPHILIC DC SYNTHONS

As an alternative to regioselective cycloaddition as described above, regioselectivity can also be achieved by stepwise ring formation, as, for example, via Michael addition of the anion of a naphthylacetonitrile such as (85) to an α,β-unsaturated ester, e.g., (86) to give an adduct (87) [154–160].



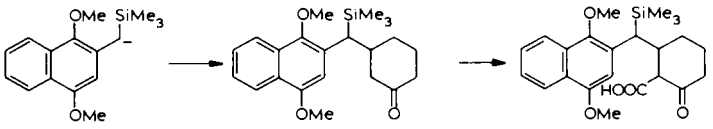
(85) (86) (87)

Hydrolysis and intramolecular acylation followed by reketalization and oxidation gives (88) which on demethylation and deketalization yields 7,9-deoxycarminomycinone (89) which can then be selectively methylated at the 4-position [156].



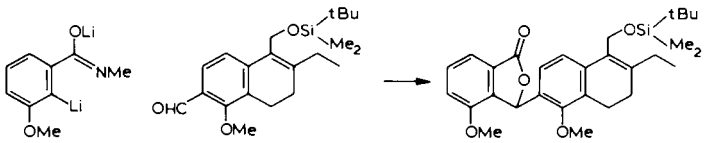
(88) (89)

This method of masking the future C12 as a nucleophilic centre can also be achieved with the anion (90) (generated by lithiation) which gives the enolate anion (91) with cyclohexenone. Then (91) can be regioselectively carboxylated to (92) with carbon oxysulphide [161], so giving access to 11-deoxyanthracyclinones.



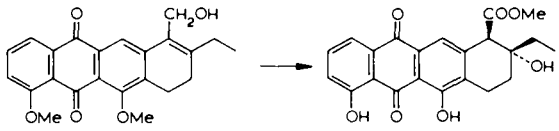
(90) (91) (92)

A DC ring nucleophile [162–172] (or indeed a BA ring nucleophile [173]) can also be generated by directed metalation. For example, lithiation of a *N*-alkyl-, or *N*-aryl- or *N,N*-dialkylbenzamide gives an *o*-metalated product such as (93) which, on reaction with an appropriate aldehyde such as (94), gives a phthalide, in this case (95) [168].



(93) (94) (95)

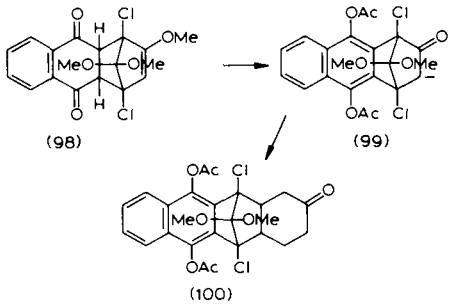
Reduction with zinc, desilylation and acid-catalyzed cyclization followed by oxidation gave (96). This was demethylated and epoxidized, the alcohol was converted to an ester function and the epoxide was stereospecifically hydrogenolysed to the single product (97) (7-deoxyaklavinone) which can be brominated and solvolysed stereospecifically to aklavinone (4) [168].



(96) (97)

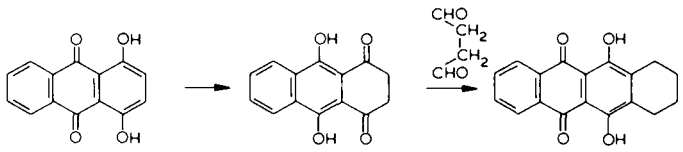
NUCLEOPHILIC DCB SYNTHONS

O'Connor and Rosen [174] used an approach in which ring A is constructed via a DCB synthon (98) generated by Diels-Alder reaction of a substituted pentadiene with naphthoquinone. Hydrolysis and acetylation followed by generation of the anion with sodium hydride gave (99), which, on reaction with methyl vinyl ketone, produced the anthracycline precursor (100).



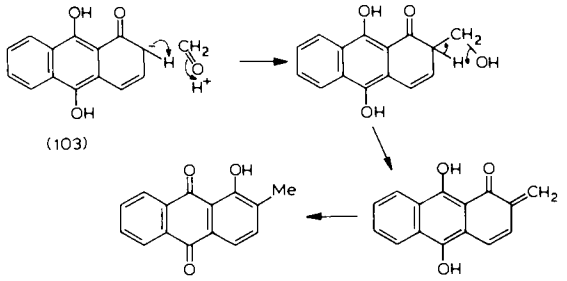
(98) (99) (100)

A more direct method of generation of DCB anions is by reduction of hydroxyanthraquinones to their dihydro (leuco) derivatives which can then undergo aldol condensation with aldehydes (the Marschalk reaction). For example, reduction of (101) to the leuco-compound (102) and reaction with succindialdehyde gives (48) [175].



(101) (102) (48)

The mechanism can be exemplified by the reaction between the anion of leuco-1-hydroxyanthraquinone (103) and formaldehyde, giving 1-hydroxy-2-methylanthraquinone (*Scheme 5.3*). By suitable adjustment of conditions it is

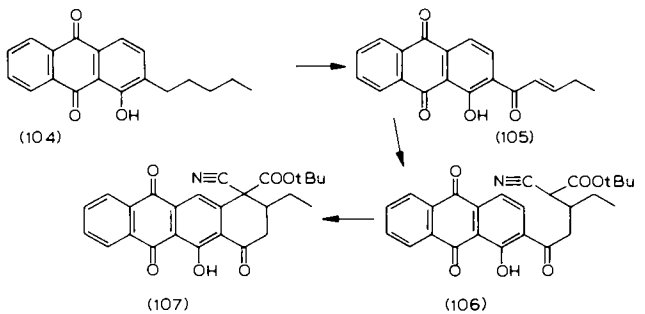


Scheme 5.3.

possible to avoid the dehydration; oxidation then yields 2-hydroxymethylanthraquinones [176].

The equivalent use of these anions in a Michael reaction has not been fully explored as yet [177], but the Marschalk reaction itself has been widely used either as an initial step or as an end step (or both) in ring A construction.

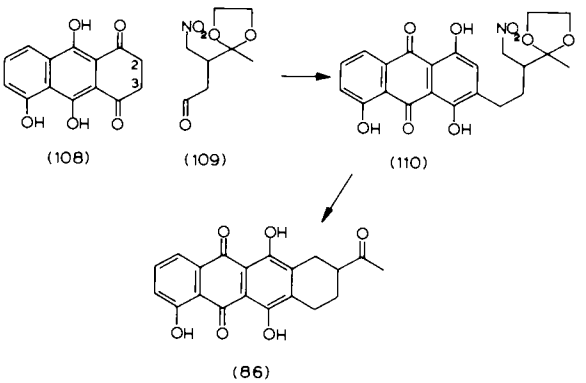
Considering first its use an initial step: with monohydroxyanthraquinones or with quinizarin (101), there is only one possible product, so further elaboration of ring A can be carried out to give, where applicable, just one regioisomer [178–181]. For example, the leuco derivative of 1-hydroxyanthraquinone (103) gave (104) on reaction with *n*-valeraldehyde; (104) was further elaborated to (105). Michael addition of *t*-butylcyanoacetate gave (106), the anion of which undergoes nucleophilic addition to the electron-deficient anthraquinone system to give (107) [180].



Use of a protected pentose as aldehyde component in reaction with leucoquinone followed by deprotection of the terminal diol, periodate oxidation

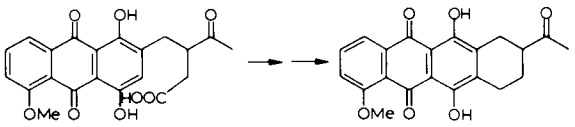
to generate an aldehyde at the previously penultimate carbon and a second (intramolecular) Marschalk reaction, produces a single stereoisomer with the defined chirality at C8 and C9 dictating the stereospecific outcome of the cycloaddition [182,183].

One feature of the Marschalk reaction is that regioselectivity can be achieved: under alkaline conditions, alkylation of 1,4,5-trihydroxyanthraquinone occurs at C2 [178,184,185]. However in a Lewis-acid-catalyzed Marschalk reaction, the 3-substituted product is formed [184,186]. Hence reaction of the leuco derivative of 1,4,5-trihydroxyanthraquinone (108) with aldehyde (109) gave compound (110), which underwent nitronate addition (cf. 106 to 107 above), and then hydrolysis gave (86) [186].



(108) (109) (110) (86)

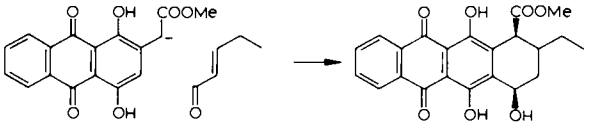
The usefulness of the Marschalk reaction as an end-reaction in A ring formation [178,187–192] is exemplified by the lack of intramolecular acylation with (111) (prepared via alkylation of 2-bromomethyl-1,4-dihydroxy-5-methoxyanthraquinone), but reduction to the aldehyde gives facile acylation to



(111) (112)

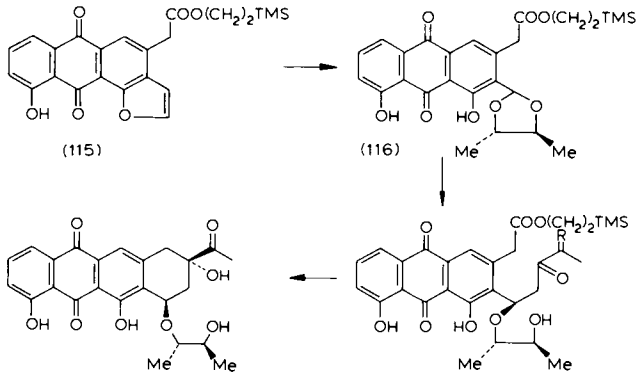
(112) by the Marschalk reaction [189]. As mentioned above, it is possible to isolate the benzylic hydroxy product [178,191,192].

A different type of DCB nucleophile derivative [193,194] is shown by Michael addition of (113) to pent-2-enal (with concomitant Marschalk-type cyclization) giving (114) [194].



(113) (114)

This approach has also found use as an end-reaction in ring A elaboration [195–199]. For example, the DCB synthon (115), prepared by Diels-Alder reaction, underwent ozonolysis and acetalization to (116). Stereospecific aldol reaction gave (117) which on ozonolysis yielded (118); cycliziation and decarboxylation then gave (119) [197].



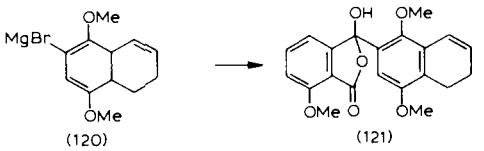
(115) (116) (119) (117) R=CH_2 (118) R=O

Acid hydrolysis of (119) led to 11-deoxydaunorubicinone, i.e., with the 'correct' stereochemistry at C7.

FRIEDEL-CRAFTS ACYLATION AND ALKYLATION

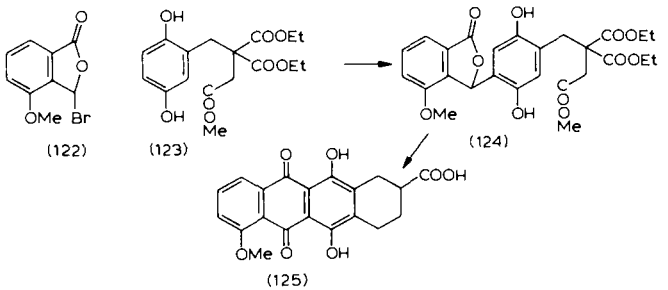
Friedel-Crafts acylation is the classical method of anthraquinone (and anthracyclinone [200]) synthesis, phthalic anhydride or phthalate hemi-esters or phthalate diesters being widely utilized [201–207] in either a double concurrent

($AlCl_3$ melt), or consecutive, process. The reaction lacks regioselectivity but this can be obtained by use of Grignard derivatives [208] such as (120) which gives (121) with 3-methoxyphthalic anhydride. Compound (121) is readily cyclized and demethylated to the dihydroxyquinone.



(120) (121)

Regioselectivity has generally been achieved by use of bromophthalides, initial Friedel-Crafts alkylation being followed by Friedel-Crafts acylation [209–212]. For example, (122) with (123) ($SnCl_4$ -CH_2Cl_2) gave (124). Selective hydrolysis and Friedel-Crafts acylation to form ring A, followed by reductive cleavage, further Friedel-Crafts acylation then oxidation, hydrolysis and decarboxylation gave (125).



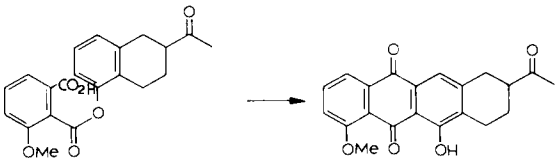
(122) (123) (124) (125)

A different, novel method for production of the intermediate phthalide [170] involves reaction of a phthalatehemialdehyde with a dihydroxytetrahydronaphthalene in presence of benzeneboronic acid.

As with the Marschalk reaction, Friedel-Crafts acylation has been used as an end-reaction in construction of ring A [213–215]; for example, during elaboration of ring A from the readily available aloe-emodin [214]. In one report, tandem Diels-Alder and Friedel-Crafts reactions were attempted but had to be carried out stepwise [216].

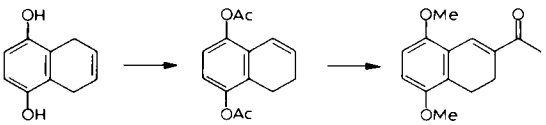
When Friedel-Crafts acylation is carried out using anhydride or ester and a hydroxynaphthalene, then initially an ester is formed which will undergo Fries rearrangement and cyclization [217,218]. For example, condensation of the

acid chloride of 3-methoxyphthalic acid hemiester with 2-acetyl-5-hydroxy-1,2,3,4-tetrahydronaphthalene gave (126) which gave (127) with boron trifluoride etherate [217].



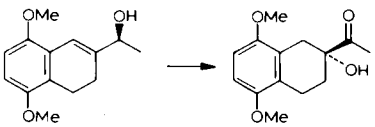
(126) (127)

Synthesis of the BA ring synthon is a prime consideration in the Friedel-Crafts approach and direct routes have been developed [219–221]; for example, (128) (from butadiene and benzoquinone) is converted to bisacetate (129) which is acylated and converted to (130) in 31% overall yield from (128) [221].



(128) (129) (130)

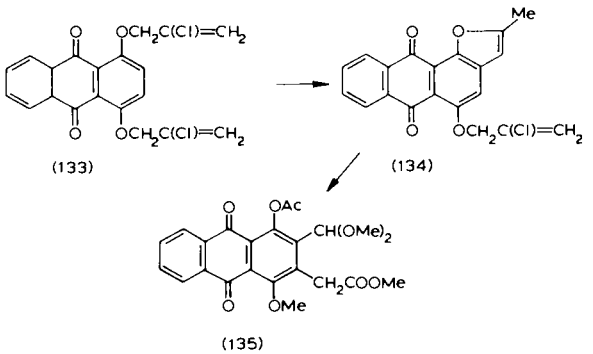
Short stereoselective routes have also been sought [222–228]. Asymmetric reduction of (130) can be achieved, giving (131), using $LiAlH_4$ partially decomposed with (1*R*,2*S*)-(–)-*N*-methylephedrine and *N*-ethylaniline. Epoxidation of (131), reductive cleavage and oxidation back to the keto group gives (132) with good retention of chirality [228].



(131) (132)

CLAISEN REARRANGEMENT

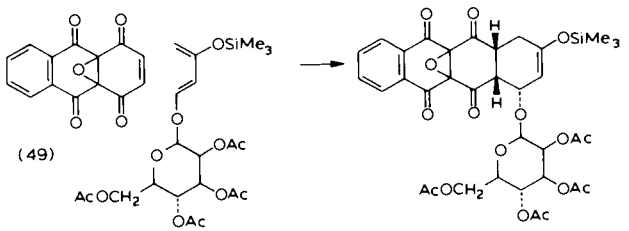
The Claisen rearrangement of anthraquinone allyl ethers has been investigated as a substitute for the Friedel-Crafts reaction or Fries rearrangement [199,229–233], but it has not proved a facile route for anthracyclinone synthesis. However, (133) from quinizarin gave (134), which could be converted to (135), a previously utilized intermediate in anthracyclinone synthesis [199].



(133) (134) (135)

SUMMARY

In the previous review in this series [11], only 14 papers on synthesis of anthracyclines were noted, with a further 14 covered in an addendum added in proof. The coverage here in this panoramic (but of necessity selective and brief) review of synthetic approaches highlights the endeavour and resourcefulness in this field, and the considerable progress made since Wong's initial total synthesis of racemic daunorubicinone [200]. In all but one approach, the strategy has been to couple the sugar as the final stage. However, Stoodley's



(49) (136) (137)

group has indicated that this may not be so in future, by showing that Diels-Alder addition of (136) to (49) gave (137) *stereoselectively*: (137) was further converted to 4-demethoxydaunorubicinone stereoselectively [234].

As with the aglycones, work on sugar and glycoside synthesis has concentrated on reducing the stages and increasing yields, for example [235–238]. L-Daunosamine has now been prepared in 10% overall yield from diethyltartrate [239] and the route from L-rhamnose to L-daunosamine has been improved [240]. Additionally, direct inversion at C3′ and/or C4′ of daunorubicin has been reported [241] and synthesis of disaccharides is being pursued [242,243]. One aspect of anthracycline glycosides is that deglycosylation can occur; to circumvent this, an attempt has been made to synthesize (stable) C-glycosides [244].

MOLECULAR BIOLOGICAL EFFECTS OF DOXORUBICIN

As doxorubicin and daunorubicin are similar both chemically and in effects, no distinction is made here between those studies using doxorubicin and those using daunorubicin unless differences between their effects are found or expected.

In the last 5 years, there has been a continued investigation of the effects of doxorubicin on nucleic acid structure and function. Also during this period there has been a rapidly growing appreciation of the metabolism, particularly reduction, of doxorubicin and its cellular consequences. More recently, the possible significance of membrane effects has been highlighted. There has also been a greater appreciation of the mechanisms of resistance to doxorubicin, of its immunostimulation properties, and of its other biochemical effects. All of these will be considered here.

BIOLOGICAL REDUCTION

There are two mechanisms of biological reduction of doxorubicin. Firstly, there is reduction of the side-chain keto group to a secondary alcohol (to give the approximately equiactive, doxorubicinol). This reduction is carried out by aldo-keto reductase enzymes [245–247] which, in humans, are of two specific types [248,249], with highest activity occurring in the liver and kidney – the enzymes utilizing NADPH + H^+ [246]. Although daunorubicin and doxorubicin bind with equal affinity, the former is a better substrate so the rate of keto-reduction is lower for doxorubicin than for daunorubicin [245].

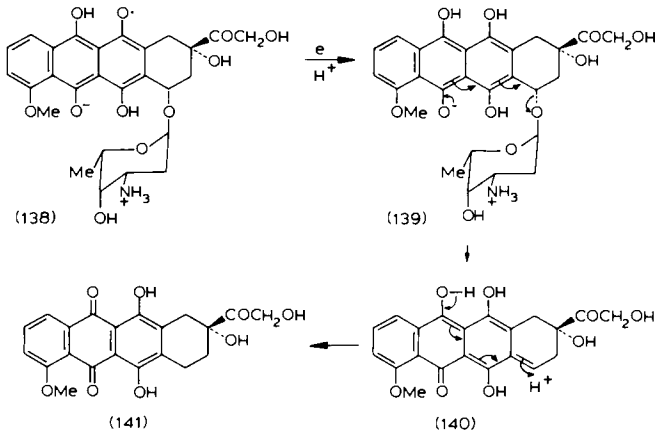
The second mechanism of reduction is a one-electron reduction to the

semiquinone, which can be detected by ESR [250–252]; its structure has recently been established as (138) [253]. The reduction can be carried out by a variety of flavo-enzymes such as NADPH-cytochrome *P*-450 reductase [254,255], xanthine oxidase [256,257], NADH dehydrogenase, succinate dehydrogenase and other enzymes of the respiratory chain [258–260]: it gives uncoupling of oxidative phosphorylation [261]. This type of reductive metabolism thus occurs in the smooth endoplasmic reticulum [254], the nucleus [262], the mitochondria [263], the sarcoplasmic reticulum of the heart [263] and the cytoplasm [263]. There is assumed to be a direct interaction of the doxorubicin with FAD or FMN in the enzyme, since doxorubicin has been shown to stack with flavin systems in solution [264,265].

The consequences of the reduction to the semiquinone depend upon the state of oxygenation. In aerobic conditions, the semiquinone is autoxidizable: the one-electron reduction potential of doxorubicin is such as to allow enzymic reduction, but is less negative than that of the oxygen-superoxide couple [253,264,265]. Hence, the electron is transferred to molecular oxygen to produce superoxide ($O_2^{\cdot -}$): other reactive oxygen species are consequently generated, such as H_2O_2 and $OH^{\cdot}$, the latter being formed either via the Haber-Weiss cycle or, more probably, by the Fenton reaction, in which $O_2^{\cdot -} + Fe^{III} \rightarrow O_2 + Fe^{II}$ and $Fe^{II} + H_2O_2 \rightarrow OH^{\cdot} + OH^- + Fe^{III}$ (the peroxide being generated from superoxide by the enzyme superoxide dismutase) [250,255,259,268–273]. On transference of the electron to oxygen, the quinone is regenerated and so can accept another electron, giving a redox cycling effect. (The superoxide produced may also interact with doxorubicin [274] possibly generating a dimer of 7-deoxydaunorubicinone.) The reaction of the semiquinone with oxygen is very rapid [275,276], so that an ESR signal for the semiquinone is not seen until all the oxygen is consumed.

When the semiquinone is generated under anaerobic conditions (or on depletion of all the oxygen) the semiquinone can reductively eliminate the sugar [245,247,255,277,278] forming the hydroquinone [139] and the nucleophilic elimination product (140), and finally the 7-deoxyaglycone (141).

Initially it was postulated that it was the semiquinone itself that underwent the elimination reaction, but it has recently been shown that the semiquinone rapidly conproportionates to the hydroquinone [259] and that this undergoes the elimination [279–283]. Indeed, it has been suggested recently that the enzymes themselves carry out a two-electron reduction [283]. One enzyme, DT-diaphorase, can only carry out a two-electron reduction, and doxorubicin may act as a substrate for such a 2*e* reduction [284,285], though this is equivocal [263]. In aerobic and anaerobic conditions, therefore, reduction of doxorubicin leads to reactive species; in the former case these are reactive oxygen species



and in the latter they are reactive reductively deglycosylated species. In either case, the diversion of electron flow will impair normal function of the enzymes; mitochondrial electron transport and cytochrome *P*-450-mediated drug oxidation, for example, will be reduced. Additionally, the reactive species can react with cellular molecules and, even in aerobic conditions, the deglycosylated active species may react locally before autoxidation occurs, particularly since such species are much more hydrophobic than parent drug due to loss of the aminosugar residue.

One well-established consequence of this generation of reactive species is lipid peroxidation [250,258,286–290]; there is a 5–7-fold increase in rat heart on administration of doxorubicin [291], and serum lipid peroxide has been shown to be elevated in experimental animals [292] and in patients [293]. Recently it has been shown that, whilst lipid peroxidation is greatest in presence of NADPH + H^+/cytochrome *P*-450 reductase/doxorubicin/Fe^{III}-ADP (lipid peroxidation requires the presence of iron [294,295]), it still occurs without the enzyme and cofactor, i.e., the doxorubicin/Fe^{III}-ADP can peroxidize lipid without metabolic reduction of the doxorubicin [294–297]. Probably a (doxorubicin)$_2$-Fe^{III}(ADP) complex is reduced to (doxorubicin)$_2$-Fe^{II} (ADP) which adds oxygen to give (doxorubicin)$_2$-$Fe^{III\text{-}O\text{-}O\cdot}$ $(ADP)_2$ which is the active species [298]. The complex probably binds to the membrane and, whereas $O_2^{\cdot-}$ is probably released, OH· is not involved [297,299,300].

Lipid peroxidation by either pathway occurs in all organs, but highest levels are in the heart, liver, kidney and spleen: the levels decline most slowly in the heart [301], the implication being that this relates to cardiotoxicity. The heart has low superoxide dismutase and catalase activity [250,258,269,302,303],

only 27% and 0.6%, respectively, of the levels in liver [304], and although some induction of enzyme synthesis occurs [302,303,305], it is insufficient to prevent toxicity. Also, glutathione levels become depleted, particularly in the heart, supporting the hypothesis of oxidative stress as a cause of cardiotoxicity [270,306–311]. The hypothesis is further supported by the reversal of cardiotoxicity by administration of coenzyme Q_{10} [292,312–316], α-tocopherol [292,306,317–320], ascorbic acid [321], and SH compounds such as cysteamine and *N*-acetylcysteine [306,309,322,323]. Selenium may also have some effect, since one form of glutathione peroxidase is a selenium-enzyme [324,325]. Considering, for example, α-tocopherol, then initially endogenous levels will be depleted and if exogenous compound is administered it will be effective until it, too, is depleted [318,326]: if given at high levels, it can cause lipid peroxidation, as it is a quinone, so vitamin B-2 butyrate has been suggested instead [327]. These compounds have no effect on the antitumour activity of doxorubicin [313,321,328,330], so the most widely held view is that the cause of cardiotoxicity is a factor, oxidative stress, not implicated in antitumour activity. The corollary is that it should therefore be possible to separate the cardiotoxic and antitumour effects: this has indeed been achieved, as we shall see later. It is the mitochondria which exhibit the first abnormal properties (swelling) in cardiotoxicity and it is suggested that it is the mitochondria, so essential for cardiac respiration, that are the toxic site of lipid peroxidation [331], this effect on the mitochondria being compounded by diversion of electrons from the electron transport chain [332].

As well as leading to lipid peroxidation, the reductive metabolism produces the reactive 7-deoxyaglycones such as (140) which will covalently bind to protein and other biological macromolecules [333,334], this is probably a general toxic effect rather than related specifically to cardiotoxicity or antitumour effect.

MEMBRANE EFFECTS

As well as causing lipid peroxidation, doxorubicin undergoes a physical interaction with membranes [335–340]. An initial ionic attraction is followed by insertion of the hydrophobic region of the molecule into the phospholipid region of the membrane [338, 341], the affinity of binding being equivalent to that for binding to DNA [338,340]. As daunorubicin is more lipophilic than doxorubicin, it penetrates further into the lipid region [342]. The interaction is non-covalent unless the lipid is peroxidized, when Schiff's base formation can occur [343]. The effect of doxorubicin insertion into the membrane is an increase in membrane fluidity [337,344,345] and this may be the cause of such

experimentally noted effects as stimulation of membrane NADH oxidase [346], the inhibition of membrane ascorbic oxidase [246], histamine release from mast cells [347], enhanced agglutination by concanavalin A [344], increased glycosylation of the cell surface [348], deacylation of phospholipid [349] and the effects on Ca^{2+} levels (see below).

Considering the membrane as a possible site of action, the membrane is indeed the first barrier the drug meets and an increase in membrane fluidity is the first observable change on treatment of cells with doxorubicin [345]. This membrane-action hypothesis has gained credibility recently by the demonstration that doxorubicin bound irreversibly to Reactigel, to agarose beads, to Dextran, to Sephadex and to PVA by a variety of coupling methods still remains active, even though the immobilized drug is not internalized [350, 351]. In these studies, care was taken to exclude leaching of drug as a possible mechanism of achieving drug levels within the cell. It appears that drug external to the cell contents is the active species. This is supported by studies with drug bound to polyglutaraldehyde microspheres; EM studies showed that drug bound to the microspheres causes membrane surface blebbing, as occurs with free drug [352,353].

Whilst doxorubicin will insert into membranes of any phospholipid composition, there is a specific binding interaction with cardiolipin, a phospholipid generally only found in mitochondria [337,339–341,343,354]. The specialized cardiolipin organization within the membrane is destroyed, the doxorubicin-cardiolipin complex segregates [355], there is an increase in rigidity of the membrane [354], and the complex can act as an electron transport system [354,356]. This action may, as well as or instead of lipid peroxidation, be the cause of the specific mitochondrial damage in heart cells [357]. However, an effect on Ca^{2+} uptake, probably mediated at the membrane level, has also been suggested as the basis for the effects of doxorubicin on mitochondria [358]. The involvement at the membrane level may explain why carnitine reverses doxorubicin cardiotoxicity [359–362].

Whether the effect on Ca^{2+} influx is due to lipid peroxidation or is a consequence of the doxorubicin-cardiolipin or doxorubicin-membrane interaction is not yet clear [287,362]. Ca^{2+} transport is affected [363–365], leading to, for example, reduction of fast-exchanging calcium in heart cells mitochondria and sarcolemmal vesicles [366–369]. Ca^{2+}-linked processes such as the Na^+/K^+ pump are impaired [365,370–373], in this case giving prolongation of the action potential [374–377]. Other consequences include inhibition of Ca^{2+}-dependent protein kinases [378], inhibition of actin polymerization [379,380], and leukotriene formation via phospholipase A_2 activation [381].

Support for the hypothesis that Ca^{2+} control impairment is related to the

cardiotoxic effects of doxorubicin is given by the reversal of the cardiotoxicity by the chelating agent, ICRF-159 and its laevo-isomer ICRF-187 [382–386], and by verapamil, propranolol and hydralazine [387].

It is hence becoming apparent that doxorubicin has multiple effects and, furthermore, that probably an effect on cardiac mitochondria is the major cause of the cardiotoxic effect. This may be mediated by lipid peroxidation and/or physical interaction with cardiolipin, coupled with a direct or indirect action on control of Ca^{2+} flux.

INTERACTION WITH THE NUCLEUS

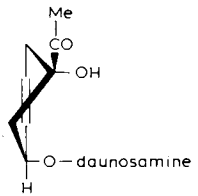
The interaction of doxorubicin with DNA is the longest-recognized biochemical effect of this drug [11]. Most of the doxorubicin in cells is in the nucleus [388–391], being accumulated up to a maximum of about 1 drug molecule per 9 basepairs [392]. Chromosome aberrations [11] and sister chromatid exchange [393] occur, and there is induction of micronuclei due to the chromosome damage [394] and crossover in mitosis [395]. Propensity for DNA damage is indicated by the ability to induce prophage λ [396]. Misreading occurs [397], so giving a mutagenic effect [398]. Compaction of chromatin occurs [399,400] and there is nucleolar segregation: initially there is formation of fibrillar centres (due to chromatin compaction), followed by segregation by redistribution of the granular component (this is where ribosomes are assembled) [401–404]. Although these effects occur to a greater extent in the heart than in other organs, they are unlikely to be the cause of cardiotoxicity [405,406], even though mitochondrial DNA synthesis is more sensitive to doxorubicin inhibition than is nuclear DNA synthesis [407].

It is well established that doxorubicin binds to DNA by intercalation between successive basepairs of the helix. This interaction has been further investigated by physicochemical techniques such as ultraviolet-visible and fluorescence spectroscopy, T-jump, stopped-flow, unwinding of closed circular DNA, thermal denaturation of DNA, electrochemical methods, spin-labelling and transient electric dichroism [408–420]. There are variations in the determined affinity constants due to differing experimental conditions (particularly variations in ionic strength) and use of the Scatchard analysis of binding data, despite its well-recognized deficiencies [421]. The affinity constant is about 2×10^6 and the number of binding sites is 0.2 per DNA phosphate: the binding of daunorubicin is marginally weaker [409,415]. Binding results in a 15° unwinding of the helix and the drug is tilted by 3° (around the short axis) and twisted by 25° (around the long axis) [418]; the helix becomes 'bent' at the site of intercalation, and stiffened [422, 423].

Binding is co-operative [424–426] but follows the neighbour-exclusion model [427] so that drug cannot bind to a basepair which already has drug bound on its other face. The drug binds only to nucleic acids of the B form and not to those of the A form [409]. The binding competition or enhancement with other molecules and ions found in biological systems has not been well studied (apart from the effect of ionic strength mentioned above), although a daunorubicin-copper complex had an enhanced binding (in contrast, Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Pb^{2+} and Cd^{2+} had no effect) [428].

As well as the intercalation reaction, there is, as with most other intercalating drugs, a weaker external mode of binding, and this is clearly shown by T-jump and stopped-flow studies [429–431].

The nature of the DNA-intercalation interaction is now clear. NMR studies of anthracyclines with polynucleotides clearly demonstrated that rings B and C are stacked with the basepairs, whereas ring D is not [432–435], suggesting the drug is oriented antiparallel to the basepairs between which it intercalates. This is in contrast to the 'classical' models for the interaction in which drug is oriented with its long axis approximately parallel to the long axes of the adjacent basepairs [11]. By the finding that binding still occurs with nucleotides with blocked major grooves, it has been shown that the sugar residue lies in the minor groove [432,436]. An X-ray crystallography study of a daunomycin/d(CpGpTpApCpG) complex has confirmed the orientation of the drug perpendicular to the plane of the basepairs with insertion from the minor groove [437]. There is zero unwinding of the helix at the intercalation site, but 8° unwinding at each of the adjacent residues. There are several stabilizing H-bonding interactions but, surprisingly, no interaction between the charged amino group and a phosphate residue. A resonance Raman spectroscopy study supports this new model of the binding [438], and insertion from the minor groove has also been suggested from a molecular mechanics energy study [439]. The conformation of the drug is close to that found in the crystal structure where, although one study suggested there were two minimum-energy conformations [440], current evidence [441–443] suggests that the minimum energy conformation accords with the structure found by X-ray crystallography for ring A [444,445] shown in (140a).



(140a)

The nature of the DNA-intercalation interaction is therefore now clear. It is less clear, however, what is the biological significance of the interaction. Certainly DNA and RNA synthesis are inhibited [11,446], with the effect on initiation being greater than on elongation – though this is by no means equivocal – and the effect on repair being less than that on replication [447–451]. Additionally, DNAase is inhibited [452,453], as is reverse transcriptase [454,455]: DNA polymerase II, which uses ss DNA, is also inhibited [456]. Most studies *in vitro* have used purified DNA rather than chromatin: drug binds to chromatin with about the same affinity but with a lower number of binding sites [457,458]. It appears that drug binds to the internucleosomal regions, leading to some change in the nucleosome organization [459,460] and to compaction of the chromatin [461,462], as noted earlier, when considering the ultrastructural changes caused in the nucleus. Possibly it is the destruction of the chromatin and nuclear organization which is the lethal event, rather than the inhibition of nucleic acid synthesis.

So far, the discussion has concerned intercalative (reversible) binding. As stated earlier, there is the propensity for generation of reactive deglycosylated drug species in the nucleus, so it is not suprising to find that covalent binding to DNA can occur [463–468]. As well as reductive activation, photo-activation of drug also can result in covalent binding to DNA [469–472] and degradation of DNA can occur [473]. Doxorubicin indeed causes single-strand (ss) breaks in DNA [474,475] and initially this was thought to be due to the capacity of the drug to generate reactive oxygen species [463,467–478], with $OH^{\cdot}$ implicated as the major interacting species [479]. However, it soon became apparent that additionally ss breaks in the DNA had protein associated with them [480–483] and could occur in absence of oxygen [484] implicating an enzymic mechanism. It is probable that intercalation into the helix leads to distortion and that a nuclease – probably a topoisomerase – then binds to relieve the strain, and becomes crose-linked, so producing double-strand breaks (most of the ss breaks are in fact ds breaks) [481,482,485–488]. Interstrand links are also formed [489].

It is probable that this damage to DNA is one of the biologically relevant effects resulting from intercalation of drug. Certainly the extent of DNA damage in leukaemic cells from patients correlates with the response to drug treatment [490,491]. This damage to DNA plus the perhaps more important effect of condensation of chromatin argue that the nuclear effects of the drug are an important, if not *the* important, effect. Cytotoxicity does correlate with nuclear drug content [492].

EFFECTS ON THE IMMUNE SYSTEM

In addition to its general immunodepressant effect, doxorubicin also has a slowly developing, discontinuous (i.e., cyclical) immunostimulant effect [493,494] mediated via a specific enrichment of a macrophage and/or natural killer cell fraction [495–500]. Daunorubicin has a lesser effect. Spleen lymphocytes are depleted [501,502] and stimulation of immature (progenitor) cells occurs, giving a consequent rise in the numbers of mature macrophages [503–506].

Whilst considering macrophages, one interesting facet is that on doxorubicin-induced degranulation of mast cells, the granules (+ drug) are taken up by macrophages which, if they subsequently interact with tumour cells, act as targetting devices [507].

OTHER EFFECTS

In addition to the above-mentioned molecular biological effects, doxorubicin has a multiplicity of other actions. It is not clear how many of these are related to or are a consequence of the above-mentioned properties. For example, the membrane effects may be responsible for the reported inhibition of uridine transport [508], of catecholamine release [509], and inhibition of creatinine phosphokinase binding to heart mitochondria [510]. The redox cycling effect may be the cause of depleted cyclic nucleotide levels [511] and of inhibition of metmyoglobin reductase [512]. Other effects include inhibition of cardiac NADP-linked isocitrate dehydrogenase [513], of enzymes of the electron transport chain (as mentioned earlier), especially succinate oxidase [514], and of guanyl cyclase [515,516]. It has been suggested that the latter in particular may be correlated with cardiotoxicity [516]. Doxorubicin also induces 3-hydroxy-3-methylglutaryl-CoA reductase, so diverting acetyl-CoA away from fatty acid synthesis to terpene synthesis [517]. Macromolecular glycolipids have been isolated which bind doxorubicin (it binds competitively with spermidine) [518] and soluble doxorubicin-binding proteins have also been identified [519,520]. The significance of these effects in the actions of doxorubicin is not yet clear.

RESISTANCE TO DOXORUBICIN

Resistance is not due to decreased DNA-binding [521–523] or to increased metabolism [524,525], but to decreased intracellular drug levels [526–528]. Drug uptake into cells, although once considered as a facilitated process [529,530], is probably by passive diffusion [531,532], whereas efflux is an

active process [533,534]. Whilst influx could be decreased in resistant cells [535], it is well established that drug efflux is enhanced in resistant cells [536,537]. The efflux system is structurally nonspecific, accommodating also Vinca alkaloids and intercalating agents other than anthracyclines [533,534]. A change in membrane structure is the likely cause of enhanced efflux [533,538,539]; the unsaturated fatty acid content increases [528] and a protein of M_r 100,000 is replaced by a new glycoprotein of M_r 180,000, which increases the hydrophilicity of the membrane surface [540–542]. As with other membrane effects already discussed, resistance seems to involve altered Ca^{2+} binding [538]; certainly, calmodulin inhibitors and Ca^{2+} antagonists such as verapamil, nifepidine, prenylamine, trifluoperazine and clomipramine reverse the resistance to doxorubicin [543–547].

Note should also be made here of decreased susceptibility to doxorubicin, for example, of cells in solid tumours compared with cells in cell culture. This may be due to less effective delivery of drug, to hypoxia, and to some of the cells being in an insensitive part of the cell cycle [548–554]. These factors are, however, not sufficient to account fully for the decreased susceptibility [555–557] and possibly there is a change in metabolic processes in the inner cells of a solid tumour [555,558]. A major concern, therefore, is the delivery of drug to the tumour cells and the need for a greater understanding of the differences between hypoxic and oxic cells. Metastases are often more sensitive to drug than the parent tumour, as there is greater accessibility and a greater rate of cell cycling [559].

DOXORUBICIN ANALOGUES

The primary aim of research on analogues is to develop improved drugs, so study of the molecular biological effects lags behind the *in vivo* testing of new compounds. Inevitably, then, most of the research has been of an empirical nature and only now are there signs that the input from molecular studies will have a major impact in analogue design. Analogues have been derived from three main sources, biosynthetic (natural) analogues (i.e., other anthracyclines), semisynthetic modification of known anthracyclines, and total synthesis of compounds modelled on the anthracyclines. For any analogue to displace doxorubicin, it must have demonstrably improved properties; several have now reached an advanced stage of clinical trial with two, mitoxantrone and epirubicin, scheduled to be marketed soon. Here, only salient features of analogues will be discussed, and no attempt is made to give a complete coverage.

ACLACINOMYCIN A (ACLARUBICIN)

Aclacinomycin A (14) is the most active of the aklavinone (4) glycosides against L1210 leukaemia, being equivalent to doxorubicin, but it is orally absorbed, less cardiotoxic and non-mutagenic and does not cause alopecia [560–565]. Its effects in enhancing the immune system are stated to be greater than those of doxorubicin [566]. It is now in phase II clinical trial as an antileukaemic agent [567]. Being more lipophilic than doxorubicin [568], cell uptake, as well as oral uptake, is greater than for doxorubicin [569–572]. Although initially reported not to be concentrated in the nucleus [573], the majority of drug is located there [574–576]. Drug binds to DNA [577–582] with an affinity constant of about $2 \times 10^6\ M^{-1}$ (i.e., approximately equivalent to that of doxorubicin). Chromosome aberrations and nuclear swelling occur, cells accumulate in the G_2 and M phases [583,584], and RNA synthesis is reduced [585]. Aclacinomycin can produce strand breaks in DNA in the presence of reducing agents [586] and is reducible to a free-radical species in biological systems [587], the 7-deoxyaglycone and the dimer of the aglycone being identified as metabolites [588].

It appears, then, that many of the effects parallel those of doxorubicin, but there are three major differences. Firstly, the lack of mutagenicity is a function of the dimethylated amine group – the *N*-didemethylated compound *is* mutagenic [564,589]. Secondly, whereas for doxorubicin the ID_{50} values for inhibition of DNA and RNA synthesis are approximately equivalent, aclacinomycin inhibits RNA synthesis preferentially [584,590–593]. Thirdly, it has been suggested from magnetic circular dichroism studies that two molecules of drug could fit into an intercalation site, side by side, with their long axes perpendicular to the long axes of the basepairs [594].

MARCELLOMYCIN

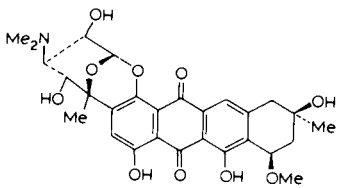
Marcellomycin (10) is a member of the 'Bohemian' group of antibiotics isolated from *Actinosporangium* sp., all the novel constituents of the group being named after characters in the opera *La Bohème* [595]. The compounds contain the ε-pyrromycinone (5) aglycone. Again, this drug affects the nucleus, giving nucleolar lesions – nucleolar segregation at low dose and microspherules in the fibrillar component at higher dose; the effects are thus different from those of doxorubicin [596]. The drug binds to DNA by an intercalative interaction [597–599] with an unwinding angle of about 10° and an affinity constant about equivalent to that of doxorubicin [599, 600]. Like aclacinomycin, RNA synthesis is preferentially inhibited [601–603]. There are thus two classes of

anthracycline, class I, like doxorubicin, with equal inhibition of DNA and RNA synthesis, and class II, with preferential inhibition of RNA synthesis. It is in fact the nucleolar RNA synthesis which is the most sensitive to class II agents [601] and the selectivity for inhibition of nucleolar RNA synthesis decreases as sugars are removed to give the disaccharide and monosaccharide derivatives – the latter showing no selectivity [602,603]. Also, selectivity is reduced when the 10-methoxycarbonyl group is removed [603]. Hence it seems that the interaction of bulky A ring substituents in the minor (?) groove of the helix confers this class II effect. Marcellomycin, too, is in clinical trial. Myelosuppression is the major dose-limiting effect; there is negligible alopecia, and no reported congestive heart failure [604].

For class II activity, the evidence suggests that a disaccharide (or higher) is required as the sugar component and that the C-10 methoxycarbonyl group should preferably be present. It must be in the correct (i.e., naturally occurring) configuration.

MENOGAROL

The natural product nogalamycin (23) [605,606] is cytotoxic, inhibiting nucleolar RNA synthesis to a higher degree than chromosomal RNA synthesis [607]. It intercalates into DNA [11,608,609], model building showing that the drug binds 'across' the helix, as does doxorubicin, with the amino-sugar in the major groove and the sugar at C-7 in the minor groove [606]. It is, however, not promising as an antitumour agent due to its toxic effects.



(141)

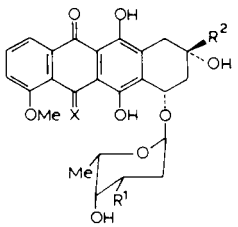
A range of semi-synthetic derivatives have been prepared and 7-*O*-methylnogarol (menogarol) (141) is the most active [605,610–613], blocking cell cycle progression, giving accumulation in the G_2 phase [614]. However, a comparison of the effects of nogalamycin and a series of analogues on RNA and DNA synthesis showed that menogarol is a poor inhibitor [611]. This is consistent with its lack of an intercalative binding to DNA [615–617], though there is

evidence for some form of interaction with DNA [616, 617] and, perhaps even more pertinent, menogarol causes ss breaks in DNA, whereas nogalamycin causes no such breaks [618]. Further work is needed to establish whether menogarol does indeed affect chromatin function and organization.

DOXORUBICIN DERIVATIVES

Compounds containing the aglycone of doxorubicin, or an aglycone closely related to it, will be discussed under this heading. Comprehensive coverage is not possible here because of the enormous number of such compounds [2,6,8,619]; however, the major groups and the most promising compounds will be discussed.

The amino group of the sugar residue is a convenient point for structural modification. Whilst *N*-acyl compounds have poor DNA-binding properties, they may still be cytotoxic [620,621]. The most extensively studied compound is AD 32 – *N*-trifluoroacetyldoxorubicin-14-valerate (142). It is more rapidly accumulated by cells than doxorubicin, is less concentrated in the nucleus, has a greater macrophage-sparing effect, is less cardiotoxic, and does not appear to act as a prodrug of doxorubicin [570,620,622,623]. It is, however, not very soluble, and the *N*-trifluoroacetyl-14-hemiadipate (AD 143) has been prepared as a more soluble AD 32 analogue [624]. AD 32 is an inhibitor of nucleic acid



Derivatives of doxorubicin and daunorubicin

	X	R^1	R^2	
(142)	O	$NHCOCF_3$	$COCH_2O(CH_2)_4Me$	AD32
(143)	O		$COCH_2OH$	CMA
(144)	N	NH_2	$COCH_2OH$	5-iminodoxorubicin
(145)	O	NH_2		rubidazone

synthesis [625–627], but it has been suggested that its effects on nucleic acid synthesis are due to inhibition of cell uptake of thymidine rather than to inhibition of nucleic acid synthesis *per se* [628]. There is still a direct effect on DNA, however, cells showing chromosomal aberrations and being blocked in the G_2 phase [625]. AD 32 causes strand breaks in the DNA, with associated DNA-protein crosslinks, as with doxorubicin [629–632]. Hence, like menogarol, AD 32 is a non-intercalating analogue capable of generating DNA damage. In contrast to *N*-acylation, *N*-alkylation leads to retention of intercalating activity [633] unless the substituents are bulky as in *N,N*-dibenzyldaunorubicin [633]. This latter compound is active *in vivo*, however, through metabolism to the *N*-monobenzyl derivative, which can intercalate [634]. The compounds, as might be expected, localize in the nucleus and cause strand scission [635,636]. Cyclic derivatives at the amino group, such as 3′-deamino-3′-(4-morpholinoyl)daunorubicin, have equivalent or greater potency as cytotoxic agents than doxorubicin [637–639]. The most exciting compound in this group is 3′-(3-cyano-4-morpholinoyl)-3′-deaminodoxorubicin (CMA) (143) which is 600-times more potent than doxorubicin vs. P388 tumour in mice, making it one of the most potent antitumour agents so far discovered [640].

As seen with AD 32, a charged amino group is not essential and some 3′-deamino-3′-hydroxy-derivatives are active [641–643]. Where the amino group is present then the compound should have the 'natural' configuration at C-3′ [644–646]. By contrast, epimerization at C-4′ does not lead to loss of activity [409,647–652]. 4′-Epidoxorubicin (epirubicin) is one of the most promising of the early doxorubicin analogues, being equiactive but less cardiotoxic [653]. Also, 4-deoxy compounds are less cardiotoxic and still active, though with reduced DNA-binding properties [654,408]. 4-Deoxydoxorubicin (esorubicin) shows perhaps even more promise than does epirubicin. 4′-*O*-Substituted compounds such as 4′-*O*-methyl and 4′-*O*-tetrahydropyranyl compounds also show activity [645,655–659]. The daunosamine sugar can be replaced with other markedly different sugar units such as glucosamine with retention of activity though loss of potency [660–663]. There has been little work, however, on non-carbohydrate substituents at C-7, though those compounds reported do have weak DNA-binding affinity [413,664,665]. The current more detailed understanding of the DNA-intercalation process could lead to developments in this area.

Moving to consideration of the aglycone, one significant modification is conversion of the 5-quinone group to a 5-imine group as in 5-iminodoxorubicin (144). This and 5-iminodaunorubicin are active, although they do not redox cycle in biological systems; this, coupled with the fact that they are much less cardiotoxic, supports the redox cycling theory of cardiotoxicity of anthracy-

clines, with the redox cycling not being important in the antitumour activity [666–669]. Whilst 5-iminodoxorubicin does not have such marked effects as doxorubicin on the nucleolus [670], it does cause protein-linked strand breaks in DNA [671] and binds to cardiolipin [672]. This confirms that it is possible to separate antitumour activity and cardiotoxicity.

Two compounds which have undergone clinical trial are carminomycin (carubicin) and 4-demethoxydaunorubicin (idarubicin); both are less cardiotoxic than doxorubicin and are orally active [673–676]. Idarubicin is more potent than the parent compound and shows equivalent DNA-binding affinity [408,677,678]. The interaction of carminomycin with DNA is weaker than that of doxorubicin [679,680], though it is a more potent inhibitor of reverse transcriptase [455].

Considering other modifications of the aglycone, 11-deoxy analogues are active [681,682], but methylation of the 6-OH or 11-OH negates DNA binding and reduces antitumour activity, consistent with the hypothesis of DNA binding being important in antitumour action [677,679,683–686]. C-7 stereochemistry is important: epimerization leads to loss of DNA-binding properties [408]. Similarly the stereochemistry at C-10 is important [687]. Whilst 9,10-anhydro compounds are less active [621,646,677,688] as are 9-*O*-methyl or 9-dehydroxy-9-methyl compounds [689,690], considerable variation at the C-9 substituents is permissible without loss of activity [646,691–693]. Hydrazones at the 13-carbonyl group, such as the less cardiotoxic rubidazone (zorubicin) (145), act as prodrugs, releasing the drug via hydrolysis [694, 695]. This lack of effect on binding of substitution in this region has been exploited to develop potential bisintercalating agents. However, since those prepared utilize a hydrazone linkage, hydrolysis may occur *in vivo* [696–698].

In summary, the considerable amount of work on doxorubicin derivatives has generated some first-generation analogues which are less cardiotoxic. In general, cytotoxicity correlates with DNA/binding properties. A comparison of the inhibitory effects of 92 anthracyclines on nucleic acid synthesis in L1210 cells confirmed the factors discussed so far [699], but disappointingly there are only two QSAR studies. It has been shown that, in C-13 hydrazone derivatives, cardiotoxicity decreases as electron-withdrawing effects increase [694]. The second study showed that activity, and cardiotoxicity, increases as hydrophilicity increases [700].

DOXORUBICIN-CARRIER COMPLEXES

An alternative approach to reduction of cardiotoxicity is to use a drug carrier to alter the disposition properties of the drug. Such drug-carrier complexes can have drug bound non-covalently or covalently. The high affinity for DNA has

been exploited to prepare (reversible) doxorubicin- and daunorubicin-DNA complexes [701–717]. Although antitumour activity is equivalent to that of free drug, the early high distribution to rapidly perfused tissue (including the heart) is reduced and up to a cumulative dose of 1200 mg m^2 of daunorubicin is possible. The daunorubicin-DNA complex dissociates before cell uptake of drug but the doxorubicin-DNA complex is endocytosed and presumably degraded in the lysosomes, so acting as a true 'lysosomotropic' agent [718].

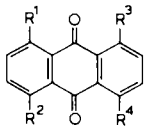
Liposomes constitute another well-researched carrier for doxorubicin [719–728]. Again, the early phase distribution to rapidly perfused tissue is reduced, so reduced cardiotoxicity is achieved, whilst antitumour activity is retained. The charge is important, it is anionic and neutral liposomes which have this reduced cardiotoxic effect. Liposomes are taken up into the cells of the RES, however; also, the anthracyclines are water-soluble so not well incorporated into liposomes, but this can be circumvented by making lipophilic derivatives [729]. Microspheres and nanospheres, for example, of albumin have also been investigated as carrier devices [730,731]. The disadvantage of all these reversible complexes is that there is no selectivity of targetting to tumour cells. This can be achieved experimentally, for example, by incorporation of antibody into liposomes [732] or by making magnetic microspheres by incorporation of magnetite [733–737], then obtaining localization by injection into an artery serving the target tissue and application of a magnet at this site.

Considering covalent coupling of drug, this has been mainly via the amino sugar, for example, to peptides, proteins and dextrans [738–741] though coupling via the C10 side-chain has also been used [742]. Mostly, metabolically irreversible linkages have been tried and drug activity has been diminished, but for example, a *cis*-aconityl spacer between drug and protein is chemically degraded at lysosomal pH [743] and a peptide linker group (of at least four amino acids) is enzymatically degraded at lysosomal pH [718,744]. As total degradation to drug may not occur, the amino acid coupled to the aminosugar should preferably be leucine, as *N*-leucyldaunorubicin retains adequate activity [745,746]. That release of drug is necessary is indicated by our studies with African trypanosomes [747–754]. Daunorubicin is trypanocidal *in vitro*, giving nuclear damage. It is not active against the organisms *in vivo*, however, in spite of achieving plasma levels and intracellular levels well in excess of levels which are trypanocidal *in vitro*. However, it *is* active when coupled to a protein, but only when the linkage is labile. Drug-protein conjugate is taken up by the organisms and the evidence is consistent with a lysosomotropic mechanism. These studies tend to argue against the membrane action hypothesis unless it is some part of the endoplasmic reticulum or lysosomal membrane which is the target site here.

As with the non-covalent complexes, specificity can be obtained by use of antibody as carrier [755–761] or by use of other specific receptors on the cell surface, for example, daunorubicin-melanotrophin for melanoma cells [762], and of daunorubicin-thyrotropin for thyroid cells [763]. Related to this concept are the reported fatty acid amides of daunorubicin which bind to the lipophilic sites for polyene fatty acids on α-fœtoprotein present on the surface of hepatoma cells [764].

ANTHRAQUINONES

The development of mitoxantrone (146), and its analogue ametantrone (147) has been reviewed in the previous volume in this series [765]. Initially we reported a series of aminoalkylamino-substituted anthraquinones such as (148) to have DNA-binding activity [766–768].



Anthraquinones

	R^1	R^2	R^3	R^4	
(146)	$NH(CH_2)_2NH(CH_2)_2OH$	$NH(CH_2)_2NH(CH_2)_2OH$	OH	OH	mitoxantrone
(147)	$NH(CH_2)_2NH(CH_2)_2OH$	$NH(CH_2)_2NH(CH_2)_2OH$	H	H	ametantrone
(148)	$NHCHMe(CH_2)_3NEt_2$	$NHCHMe(CH_2)_3NEt_2$	H	H	
(149)	$NH(CH_2)_2NEt_2$	H	H	H	
(150)	$NH(CH_2)_2NEt_2$	$NH(CH_2)_2NEt_2$	H	H	
(151)	$NH(CH_2)_2NEt_2$	H	a	H	
(152)	$NH(CH_2)_2NEt_2$	H	H	a	

[a] $NH(CH_2)_2NEt_2$

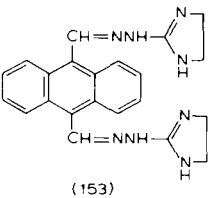
Random screening [769] and synthesis of analogues of these compounds [770] led to ametantrone, thence to mitoxantrone [769,770]. Only the most recent studies will be discussed here. Mitoxantrone has a spectrum of activity similar to that of doxorubicin, but has a higher therapeutic index [771]. The major dose-limiting effect is myelosuppression [772–777]: it does not show the same type of ECG changes as with doxorubicin [778–780]. Whilst some

patients have shown cardiotoxicity, they have generally previously been treated with other anthracyclines [781,782]. So, whilst the potential for cardiotoxicity has yet to be defined exactly, it is clear that mitoxantrone has very much reduced (and may be negligible) cardiotoxicity compared with doxorubicin. Mitoxantrone gives effects on the nucleus similar to those of doxorubicin, as for example chromatid gaps, breaks and sister chromatid exchange, and it binds intercalatively to DNA [783–785].

The development of mitoxantrone is a milestone in anthracycline research, not least because the anthraguinones are more readily accessible synthetically than anthracyclines. Of the recent analogues reported [786–789], the structural isomer with 5,6-hydroxy groups (rather than 5,8-) shows equivalent or greater activity in experimental tumour systems [788]. Two aspects are of further note. Firstly, mitoxantrone is unstable in plasma [790]; the relevance of this is not yet clear. Secondly, the increased knowledge of the DNA-intercalation process allows the possibility of more rational drug design. To this end, we have now studied the interactions of (149)–(152) by computer graphics simulation of their docking into an intercalation site (with intermolecular mechanics calculation of the fit) and by experimental analysis of the interaction with DNA in solution [791–795]. Ranking of the compounds in order of their experimentally determined affinity for DNA correlated with the ranking from the graphics modelling. The latter showed that the preferred interaction of (149) and (150) was 'across' the helix, whereas (151) and (152) intercalated with their long axes parallel to the long axes of the basepairs. Compound (152) has the highest affinity for DNA and graphics modelling shows that DNA breathing (transient basepair unstacking) must occur to allow insertion into (and release from) the intercalation site. Results from kinetic studies are consistent with this, and the rate of dissociation of (152) from DNA intercalation is slower than for the other compounds or for doxorubicin and daunorubicin.

The possibility of one- or two-electron reduction of mitoxantrone and ametantrone *in vivo* needs to be considered, particularly as they have been shown to undergo stacking with FMN [796]. We have shown that free radicals can be formed from several anthraquinones in mouse liver subcellular fractions [797]. As with doxorubicin, free-radical signals were also obtained by ESR from mitoxantrone and ametantrone under anaerobic conditions as pH 8.2 in mouse liver subcellular fractions [798], mitoxantrone also giving a signal at pH 7.5. Further, mitoxantrone has also been shown to be reduced by cytochrome *P*-450 reductase, whereas ametantrone binds but is not reduced [799,800]. Although doxorubicin depleted hepatic glutathione *S*-transferase activity and gave hepatic lipid peroxidation in treated mice, ametantrone had no effect here, though both drugs depleted cytochrome *P*-450 activity [801].

Similarly, in drug-treated mice, doxorubicin gave depletion of glutathione and lipid peroxidation in the heart, whereas ametantrone actually inhibited cardiac lipid peroxidation; this is consistent with the lack of increase of oxygen consumption in liver microsomes by ametantrone [802]. This lack of stimulation of lipid peroxidation is shared by both ametantrone and mitoxantrone [803,804] and they inhibit doxorubicin-stimulated lipid peroxidation [803,805–807]. Hence with ametantrone, binding to the enzyme occurs but drug is not reduced. With mitoxantrone, one-electron reduction or perhaps two-electron reduction [808] occurs, but there is no redox cycling under biological conditions. The fact that mitoxantrone and ametantrone show no or negligible cardiotoxicity supports the theory of the redox cycling properties of anthracyclines being the cause of the cardiotoxicity.



(153)

Finally, although not an anthraquinone, mention should be made of bisantrene (153) [809] which has nuclear, DNA-binding and DNA-damage effects similar to those of doxorubicin and mitoxantrone [810,811]. Leucopenia is the major dose-limiting effect and no cardiotoxicity has been reported but phlebitis is common [812–815]. In one study in experimental systems, the following ranking of activity was found: mitoxantrone > doxorubicin > bisantrene [816].

CONCLUSION

Whilst a wide range of recent work on doxorubicin and its analogues has been covered here, the fact that this review is not exhaustive serves to illustrate the vast amount of research carried out since this series' last review of the field [11] 6 years ago. In that review, it was stated that there was a need for a rationale to select compounds for testing by predicting active but non-cardiotoxic structures. As seems almost inevitable from the history of medicinal chemistry to date, new potentially clinically useful analogues have in fact arrived by screening of natural products, or by chemically-based intuitive semisynthesis

and synthesis, rather than by truly rational design. Only recently has it become apparent that full rational design could be a realizable goal in the future. One factor that has frustrated rational analogue design to date in the lack of identification of which one (or more) of the cellular effects of doxorubicin discussed above is the primary lethal event. It does seem probable, however, that the effects on the nucleus will be a major determinant in cytotoxicity. Furthermore it seems clear that cardiotoxicity is a consequence of the biological reduction. The separation of antitumour and cardiotoxic effects hoped for in the previous review [11] has been achieved in several analogues. A number of such first-generation analogues are in clinical trial. At present, the parents are still thriving and we are now awaiting the arrival of the 'child prodigy' [817]. Even if one of these first-generation compounds turns out to be the 'child prodigy', the full potential of the anthracyclines in cancer therapy still will not have been fulfilled. Reduction of myelosuppressive and other toxic effects is a further goal for anthracycline research. It is to be hoped that in another 6 years' time it will be possible to report that second- or third-generation analogues have made significant inroads in this area.

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6 Antithrombotic Assessment and Clinical Potential of Prostacyclin Analogues

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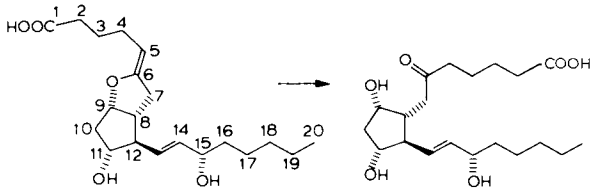
INTRODUCTION

The discovery that blood vessels synthesize and release an unstable arachidonic acid metabolite, prostacyclin (PGI_2) which is a potent vasodilator and inhibitor of platelet aggregation [1], has implicated this prostanoid as an important factor in the regulation of vascular tone and haemostasis [2]. This bicyclic enol-ether (1) is derived from the fatty acid precursor arachidonic acid via the unstable endoperoxide intermediates. These are transformed by the



1 2

action of a further enzyme system, termed prostacyclin synthetase, which is located predominantly in vascular endothelium. Prostacyclin is unstable at physiological temperatures and pH, and readily undergoes chemical hydrolysis to form its relatively inactive decomposition product, 6-keto-$PGF_{1\alpha}$ (2). It has become apparent that prostacyclin has many potential clinical applications for the management of thromboembolic and other vascular disorders.

Prostacyclin has proved valuable in preventing platelet aggregation during interaction of blood with the artificial surfaces of extracorporeal circulatory systems. Thus, prostacyclin improves haemocompatibility during charcoal haemoperfusion in dogs [3] and in patients with fulminant hepatic failure [4,5]. Experimental studies in dogs suggested that prostacyclin could be used in conjunction with or as an alternative to heparin for haemodialysis [6]. This heparin-sparing action of prostacyclin has been demonstrated in patients with chronic renal failure undergoing regular dialysis therapy [7], while prostacyclin was used as the sole anti-thrombotic agent in a further study in patients on long-term dialysis [8]. In cardiopulmonary by-pass experiments in dogs using a bubble oxygenator, prostacyclin in combination with heparin preserved both platelet number and function with minimal fibrinogen consumption and deposition on the arterial filters [9]. Preservation of platelet number and function by prostacyclin during extracorporeal oxygenation with a membrane has likewise been demonstrated [10]. Prostacyclin has also been evaluated in several double-blind clinical studies on cardiopulmonary bypass [11–13],

where preservation of platelet number and function was observed, with a reduction in blood loss in the first 18 h after the operation. Prostacyclin infusion has also been demonstrated to decrease significantly platelet deposition on vascular prosthetic grafts [14].

Prostacyclin has potential in the clinical treatment of peripheral vascular disease. In open studies, local intra-arterial administration of prostacyclin led to alleviation of pain, regression of necrosis and healing of ulcers in cases of advanced arteriosclerosis obliterans in man [15,16]. More recent double-blind studies have demonstrated that prostacyclin is effective in the treatment of Raynaud's syndrome [17], and reduces ischaemic rest pain in severe arterial disease [18].

Prostacyclin could also be useful in other situations where excessive platelet aggregation takes place, such as in conditions of thrombotic thrombocytopenic purpura or haemolytic uraemic syndrome and the platelet component of the rejection process during transplant surgery [16]. Preliminary studies on the use of prostacyclin in severe idiopathic pulmonary artery hypertension [19] have also been reported. In addition, there is preliminary information suggesting that prostacyclin might be useful in the treatment of congestive heart failure [20] and in improving the clinical development of stroke [21]. However, such potential therapeutic applications are at an early stage and further work is needed before assigning a definite therapeutic role for prostacyclin for such utilities.

REQUIREMENTS FOR PROSTACYCLIN ANALOGUES

Prostacyclin (epoprostenol) is now available for clinical use as the freeze-dried sodium salt. When mixed with the diluent buffer (sodium chloride 0.15% (w/v) and glycine buffer solution 0.19% (w/v); pH 10.5), the prostacyclin solution will remain stable over 24 h when stored at 2°–8°C. However, there is much interest in synthesizing a chemically stable analogue which has a biological profile comparable with that of the parent prostacyclin. Furthermore, one of the most important aims of the pharmacologist and chemist working in this area is the definitive separation of the platelet and vascular activities in an orally active synthetic prostacyclin analogue, both for use as a research tool and as a clinically useful agent.

Since the time of the elucidation of the chemical structure of prostacyclin (1) as (5*Z*)-9-deoxy-6,9α-epoxy-Δ^5-PGF_1 following a collaborative study between the Wellcome Research Laboratories and the Upjohn Company in 1976 [22], many synthetic prostacyclin analogues have been prepared and reported. However, many of the analogues described in the chemical literature

are devoid of the biological activity characteristic of prostacyclin, namely, inhibition of platelet aggregation and cardiovascular activity. Furthermore, many of the more chemically oriented publications on prostacyclin analogues provide scant details of the biological profile of the compounds and the methods employed in their evaluation, thus making structure-activity comparisons between analogues and publications somewhat difficult.

There have appeared several excellent reviews on the chemical synthesis and more chemical aspects of prostacyclin analogues and their derivatives, to which the reader is referred [23–26]. The current paper attempts to review and assess the biological activity of the more potent prostacyclin analogues reported over the last 6 years, with particular reference to their potential antithrombotic utilities.

PHARMACOLOGICAL EVALUATION – METHODOLOGICAL ASPECTS

The primary pharmacological activity of prostacyclin presently of major interest to the pharmacologist in the development of their antithrombotic potential is its ability to inhibit platelet aggregation both *in vitro* and *in vivo*.

STUDIES ON PLATELET AGGREGATION *IN VITRO*

Preliminary evaluation of the anti-aggregating potency is usually carried out in plasma using a turbidometric aggregometry system developed by Born [27] which determines the optical density of the plasma; as platelets aggregate, the optical density of the plasma decreases. This change in light transmission is detected by a photocell system, and the resulting chart trace can be accurately quantified. Using this system, typical sigmoid dose-response curves can be constructed and subjected to analysis.

In our laboratory [28], human blood is freshly collected into plastic vessels containing trisodium citrate (3.15%; 0.1 vol. with 0.9 vol. blood) and centrifuged ($200 \times g$ for 15 min) at room temperature. The platelet-rich-plasma (PRP) is withdrawn into plastic containers, closed and kept at room temperature. Inhibition of platelet aggregation by prostanoids is determined in a Born-type optical aggregometer by incubating aliquots (0.5 ml) of the PRP for 1 min at 37°C (stirred at 900 rpm) with or without the inhibitor prior to addition of sufficient adenosine diphosphate (ADP) to just cause maximal aggregation. Dose-inhibition curves are constructed for each compound and the ID_{50} value is calculated as the dose required to reduce the aggregation

to 50% of its control amplitude. The standard, prostacyclin (as the sodium salt), is freshly dissolved in 1 M Tris buffer (pH 9.6 at 4°C) and stored on ice; subsequent dilutions are made with ice-cold Tris buffer (50 mM, pH 8.6) or in isotonic sodium bicarbonate solution (1.25% (w/v); pH 8.6) and used immediately.

Comparable studies on the anti-aggregating potency in human PRP can be conducted against platelet aggregation induced by other agents including arachidonic acid, collagen, thrombin and thromboxane mimetics (for example, U-46619; 11α,9α-epoxymethano-PGH_2). Furthermore, PRP obtained from other species including rat, rabbit, dog, horse, guinea-pig and monkey can be used, although human platelets are the most sensitive to the inhibitory actions of prostacyclin (*Table 6.1*) as well as its analogues. The order of sensitivity to prostacyclin is human > dog = guinea pig > rat > rabbit > horse = sheep [28]. Use of PRP from several species to compare the shape of the dose-response with an analogue to that of prostacyclin can provide useful information on the nature of the interaction of the analogue with prostacyclin-binding sites on platelets.

Since stimulation of platelet adenylate cyclase with the resultant increase in cyclic AMP is considered to be the primary and fundamental event underlying platelet inhibition by prostacyclin, it can be useful to correlate the platelet anti-aggregating potency *in vitro* of prostacyclin analogues with their ability *in vitro* to activate platelet adenylate cyclase and to elevate platelet cyclic AMP levels.

Table 6.1. INHIBITION OF ADP-INDUCED PLATELET AGGREGATION IN PLASMA FROM SEVERAL SPECIES BY PROSTACYCLIN (PGI_2), PGD_2, PGE_1 AND 6-KETO-$PGF_{1\alpha}$

Results, expressed as the dose causing 50% inhibition (IC_{50}), are the mean ± S.E.M. of 3–20 experiments for each value (data derived from Ref. 28 and from Hamid and Whittle, unpublished work).

	IC_{50} (*ng/ml*)			
	PGI_2	*PGD_2*	*PGE_1*	*6-keto-$F_{1\alpha}$*
Man	0.4 ± 0.1	11 ± 2	21 ± 3	282 ± 37
Dog	0.8 ± 0.2	970 ± 300	9 ± 3	7130 ± 1100
Guinea-pig	0.8 ± 0.2	200 ± 20	20 ± 3	5000 ± 500
Rat	1.7 ± 0.5	≈ 50,000	8 ± 1	5200 ± 1200
Rabbit	2.8 ± 0.5	370 ± 50	16 ± 3	7830 ± 1160
Sheep	3.7 ± 0.8	16 ± 5	37 ± 8	8750 ± 1250
Horse	3.7 ± 0.5	27 ± 6	39 ± 10	6625 ± 1460

Inhibition of platelet aggregation by prostacyclin and analogues in citrated whole blood *in vitro* can be determined in the electronic aggregometer, where platelet aggregation is assessed by use of an impedance electrode system [29]. The electrical resistance between two closely placed platinum electrodes, which initially become coated with a monolayer of platelets, increases when aggregating platelets deposit on these probes. This system gives aggregation traces comparable with those obtained in an optical aggregometer (although without shape-change information) and ID_{50} values can be calculated in an analogous fashion. Studies in whole blood can also be conducted by an *in vitro* platelet counting technique, utilizing an Ultra-flow whole-blood platelet counter [30,31]. Aggregation by ADP or collagen is induced in samples of whole blood, shaken or stirred at 37°C, and aliquots removed at various time intervals for counting of free platelets. The degree of aggregation is calculated from the fall in free platelet count, and thus inhibition of aggregation can be readily determined. Such studies in whole blood ensure that binding of the analogue to blood components will not alter their anti-aggregating activity. Furthermore, studies using washed platelets, resuspended in albumen-free Tyrode's solution, can be employed to investigate any effect of binding of the analogues to plasma protein [31].

Although the basic methodology used by most investigators for the *in vitro* evaluation of prostacyclin analogues as inhibitors of platelet aggregation is comparable, changes in experimental protocol (for example, in the concentration of agonist used to induce aggregation) may alter the activity of the compounds. Furthermore, the absolute potency of prostacyclin may vary between laboratories, perhaps reflecting the instability of this reference standard, and will thus influence the data for the relative potency of various stable analogues. To allow a more rigorous comparison of the anti-aggregating activity of many of the prostacyclin analogues discussed, we have therefore evaluated a number of representative analogues, both stable and unstable, in our own experimental systems. These prostacyclin analogues and derivatives were synthesized and supplied by the Upjohn Company, Kalamazoo.

STUDIES ON CARDIOVASCULAR ACTIVITY

The cardiovascular activity of prostacyclin analogues is a useful rapid assessment of their pharmacological potency and duration of activity *in vivo*. Furthermore, the vasodilator properties of prostacyclin analogues may be of therapeutic value for clinical use. In our preliminary evaluation of cardiovascular activity [32], anaesthesia is induced in male Wistar rats (250–300 g body weight) or male rabbits (2–2.5 kg) with sodium pentobarbitone (30 mg kg^{-1},

i.v.) and maintained with supplements (3 mg kg^{-1}). Arterial pressure is recorded from a cannulated femoral artery and heart rate derived by integrating the arterial pulse. Rectal temperature is maintained at 37°C by thermistor-controlled radiant heat. Each compound is injected or infused into a femoral vein in a volume of 0.2 ml and flushed in with 0.2 ml of saline (0.9% w/v). Compounds can also be administered intra-arterially, via a catheter inserted retrogradely into the left carotid artery to reach the aortic arch.

Dose-response relationships for the fall in mean systemic arterial blood pressure (BP) with the analogues are constructed and compared with those obtained with prostacyclin in the same animal, and a potency ratio calculated. An index of the duration of the hypotensive effect for each compound can be obtained by measuring time from the peak response to when it had recovered to half its peak response ($T_{1/2}$ min) for a standard fall in BP.

Comparable studies in other species can be conducted in a similar fashion, while the local vascular activity in specific organs and vascular beds including the pulmonary and cardiac circulation and gastric, renal and splanchnic vascular beds following local intra-arterial administration of prostacyclin analogues can also be monitored. Changes in local blood flow to a vascular bed can be detected via an electronic flow-probe or by changes in vascular resistance derived from perfusion pressure measurements under conditions of constant flow perfusion [33].

Some indication of the vascular activity can also be gleaned from *in vitro* studies (where metabolism of the compounds may be minimal) utilizing isolated strips of vascular tissue suspended in an organ bath or in cascade superfusion in which changes in tension are detected by transducer systems. Isolated tissues which are useful for determining the spasmogenic activity of prostacyclin analogues include strips of rabbit coeliac and mesenteric artery and bovine coronary artery [34,35]. Alternatively, the vascular activity of the analogues *in vitro* can be determined in isolated whole organs, including rat and rabbit heart, kidney and stomach, perfused with physiological salt solutions.

STUDIES ON PLATELET AGGREGATION *EX VIVO*

Studies on the potency of various prostacyclins as inhibitors of platelet aggregation *in vitro* and on cardiovascular parameters *in vivo* provide useful information on their biological profile. However, it has become clear that for many studies on selectivity of action of prostacyclin analogues, these parameters should be assessed simultaneously in the same animal. Therefore, we have developed a technique whereby the inhibition of platelet aggregation *ex vivo* and concurrent cardiovascular changes can be determined, and this has

been employed in studies in anaesthetized rabbits, dogs and monkeys as well as in man [32,36]. To enable a more rapid preparation of samples of PRP so that platelet function could readily and continually be assessed (especially during the administration of labile substances such as prostacyclin), a rapid-spin method for the centrifugation of blood samples has been developed [32]. This 'rapid-spin' method enables platelet aggregation to be assessed in platelet-rich plasma (PRP) prepared within 1.5 min of withdrawing the blood sample from the animal (*Figure 6.1*). This technique, which allows continual monitoring of platelet aggregation throughout periods of drug infusion, appears particularly useful in the study of labile or rapidly metabolized substances, whose plasma half-life may be far shorter than the time taken to prepare PRP by conventional methods (15–20 min).

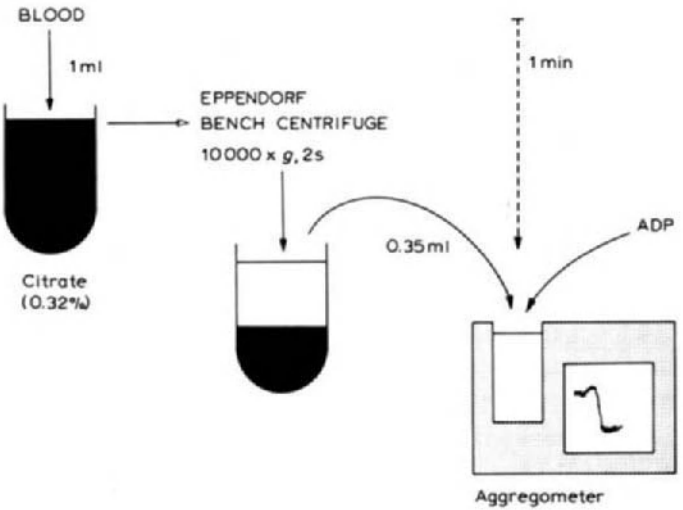


Figure 6.1. Rapid preparation of platelet-rich plasma by a quick-spin technique and determination of its aggregation induced by ADP.

Male rabbits (2–2.5 kg body weight) are anaesthetized with sodium pentobarbitone and systemic arterial blood pressure (BP) is recorded from a cannula filled with heparinized saline (5 units/ml) in a femoral artery; no heparin is administered to the animal. Drugs are administered via a cannula in the jugular vein. Blood samples (3.0 ml) are slowly collected into a plastic syringe containing trisodium citrate (3.18%, 0.1 vol. to 0.9 vol. of blood) from a cannula inserted into the femoral vein, shaken gently and transferred to two Eppendorf plastic tubes (1.5 ml each). Each tube is spun separately in a modi-

fied Eppendorf centrifuge for 2 s (maximum centrifugal force, 10,000 × *g*). The PRP from each tube is collected separately and 0.4 ml aliquots are transferred to the aggregometer and incubated at 37°C for 1 min prior to addition of sufficient ADP (15 μM) to produce near-maximal aggregation. The time-interval between removal of blood samples and the transference of the PRP to the aggregometer is only 1 min (*Figure 6.1*).

Studies on the inhibition of dog platelet aggregation can be carried out in comparable fashion using blood samples from chloralose-pentobarbitone-anaesthetized dogs. Blood samples (6 ml) are collected from the left femoral artery into trisodium citrate (0.318% final concentration), PRP prepared by the rapid spin method and aliquots are transferred to an aggregometer; platelet aggregation is induced by ADP (5–20 μM). Systemic arterial blood pressure can be recorded from a carotid or femoral artery and drugs administered via a femoral or jugular vein.

Other *ex vivo* techniques which have been utilized in the assessment of prostacyclin and its analogues include the isolated tendon preparation where blood from a cannulated artery of cat or rabbit is continually superfused over a strip of achilles tendon. This tissue increases in weight (detected by a sensitive electronic transducer) during superfusion due to the deposition of platelet aggregates from the blood on the exposed collagen surfaces. Prevention of platelet aggregation or disaggregation following infusion of the compounds either intravenously or directly into the superfusing blood can thus be assessed [37].

STUDIES ON PLATELET AGGREGATION *IN VIVO*

Prevention of the platelet-derived thrombus in the carotid artery at the site of local electrical damage has been demonstrated with prostacyclin. In these studies, the anode was placed on the exposed carotid artery of the anaesthetized rabbit, and a current of 2 mA was passed for 3 min, and 30 min later the artery was removed for histological inspection of the thrombus formation [38]. Changes in bleeding time, which depend on platelet behaviour at the site of a standard superficial cut in the ear of the anaesthetized rabbit, or on the forearm of human volunteers, have also been employed to detect potential anti-thrombotic activity [38].

Prostacyclin has been demonstrated to inhibit the platelet-derived thrombus formation in the exposed microcirculation of the hamster cheek pouch. Thrombus formation was induced by the iontophoretic application of ADP to the external surface of the superfused microvessels, and the time taken for a thrombus to form and detach from the vessel wall determined by direct microscopic study [39].

An *in vivo* model of platelet-induced coronary occlusion following partial obstruction of the exposed circumflex coronary artery with a snare ligature has also been utilized to study the activity of prostacyclin and analogues in preventing such intravascular aggregation in the anaesthetized dog [40].

A further technique for the evaluation of prostacyclin actions on platelet aggregation *in vivo* has been the use of the 'Hornstra filter-loop' in the dog [41]. In this system, the deposition of platelets on a nickel micromesh screen placed in an arterial circuit was determined by the increase in pressure across the screen. An experimental model for thromboembolism utilizing a femoral shunt in baboons has also been developed [42]. The consumption of platelets was found to be dependent on the surface area of the shunt and the materials employed in its construction. Using this model, both inhibition of platelet consumption and cardiovascular changes by prostacyclin derivatives can be determined [42]. The action of a prostacyclin analogue on bleeding time has also been determined by the use of an arterial-venous shunt in the rat, in which the time taken for a standard-size hole made in the polyethylene tubing to be occluded was measured [43].

Intravascular platelet aggregation in anaesthetized rats or guinea-pigs has been determined by the measurement of the free platelet count in blood continuously perfused through a detector cell in a whole-blood platelet counter. Aggregation was induced by the intravenous infusion of ADP or collagen, and a dose-related fall in platelet count was determined [44]. In a further *in vivo* model to determine the anti-aggregation actions of prostacyclin and its analogues, indium-labelled platelets were infused into anaesthetized guinea-pig and radioactivity in the thoracic and abdominal regions was detected by collimated scintillation probes. Intravenous injection of ADP increased the ratio of radioactivity between thoracic and abdominal regions, an index of the pulmonary entrapment of aggregated platelets [45].

UNSTABLE AND MORE STABLE ANALOGUES AND DERIVATIVES

Early studies indicated that the methyl esters of prostacyclin and its analogues were more resistant than was the free carboxylic acid to chemical hydrolysis in aqueous solution [46]. Although the potency of prostacyclin methyl esters as inhibitors of human platelet aggregation was reduced [42], we have observed that their activity increased on incubation with either PRP or platelet-free plasma. This presumably reflects the enzymatic hydrolysis of the methyl ester by the esterases found in human plasma. Since plasma from rats

contains a high level of such esterases, such hydrolysis of these esters may occur more avidly in studies using rat plasma. Similarly, such esters of prostacyclin may show a higher level of activity as vasodepressors following de-esterification *in vivo* than would have been predicted from *in vitro* studies with isolated vascular strips.

In our studies on human platelet aggregation, the (5*E*)-isomer of prostacyclin had reduced anti-aggregatory activity in PRP, being 0.05-times as active as the natural configuration, but was more active than the 19 hydroxy-, the (11*S*)- or the 15-deoxyprostacyclins, the latter being essentially devoid of anti-aggregatory activity (*Table 6.2*). The (15*S*)-15-methyl derivative of prostacyclin was some 10-fold less active than prostacyclin *in vitro*, but was more active than the 16,16-difluoroprostacyclin and considerably more potent than 16,16-dimethyl prostacyclin (*Table 6.2*). Both 16- and 17-phenyl derivatives showed anti-aggregating activity in the same range as the 15-methyl-substituted analogue (*Table 6.2*).

Table 6.2. INHIBITION OF ADP-INDUCED PLATELET AGGREGATON IN HUMAN PLASMA BY PROSTACYCLIN AND ITS DERIVATIVES

Results, expressed as IC_{50} (concentration causing 50% inhibition) following 1 min incubation at 37 °C, are shown as mean ± S.E.M. of at least four experiments for each.

	IC_{50} (ng ml^{-1})	Relative potency
PGI_2	0.4 ± 0.1	1
(5*E*)-PGI_2	7.3 ± 1.3	0.05
(11*S*)-PGI_2	370 ± 80	0.001
(19*RS*)-19-Hydroxy-PGI_2	44 ± 10	0.009
20-Methyl-PGI_2	1.0 ± 0.2	0.4
20-Ethyl-PGI_2	0.4 ± 0.2	1
20-Methyl-PGI_2 methyl ester	19 ± 2	0.02
20-*N*-Pentyl-PGI_2	2.2 ± 0.6	0.18
2-Nor-PGI_2	23 ± 4	0.02
PGI_3	0.7 ± 0.2	0.57
(15*R*)-PGI_2	17 ± 2	0.02
(15*R*)-PGI_2 methyl ester	1100 ± 100	0.0004
15-Deoxy-PGI_2	> 2000	< 0.0002
(15*S*)-15-Methyl-PGI_2	4.3 ± 0.5	0.09
16,16-Dimethyl-PGI_2	40 ± 6	0.01
16,16-Difluoro-PGI_2	12 ± 4	0.03
16-Phenyl-PGI_2	5 ± 2	0.08
17-Phenyl-PGI_2	11 ± 4	0.03

Table 6.3. INHIBITION OF ADP-INDUCED HUMAN PLATELET AGGREGATION BY PROSTACYCLIN, ITS METABOLITES AND DERIVATIVES *IN VITRO*

Results, given as the IC_{50} value (concentration causing 50% inhibition) following 1 min incubation at 37°C in PRP and the relative potency to prostacyclin, are the mean ± S.E.M. from at least four experiments. Data from Ref. 48 and unpublished work.

	IC_{50} (ng ml^{-1})	*Relative potency*
PGI_2	0.4 ± 0.1	1
6-Keto-$PGF_{1\alpha}$	282 ± 37	0.0014
15-Keto-PGI_2	> 2000	< 0.0002
(15*R*)-6-Keto-$PGF_{1\alpha}$	> 2000	< 0.0002
13,14-Dihydro-6-keto-$PGF_{1\alpha}$	> 2000	< 0.0002
13,14-Dehydro-6-keto-$PGF_{1\alpha}$	757 ± 58	0.0005
13,14-Dihydro-PGI_2	100 ± 20	0.004
6,15-Diketo-$PGF_{1\alpha}$	> 2000	< 0.0002
13,14-Dihydro-6,15-diketo-$PGF_{1\alpha}$	> 2000	< 0.0002
(11-epi)-6-Keto-$PGF_{1\alpha}$	> 2000	< 0.002
6-Keto-PGE_1	6 ± 0.7	0.07
6-Hydroxy-PGE_1	703 ± 72	0.0006

The metabolites of prostacyclin and its breakdown product, 6-keto-$PGF_{1\alpha}$, which would be produced *in vivo* following enzymatic attack by 15-hydroxy-prostacyclin dehydrogenase (15-PGDH; which oxidizes the 15-hydroxy grouping to 15-keto) or by Δ^{13-14} reductase or a combination of the two

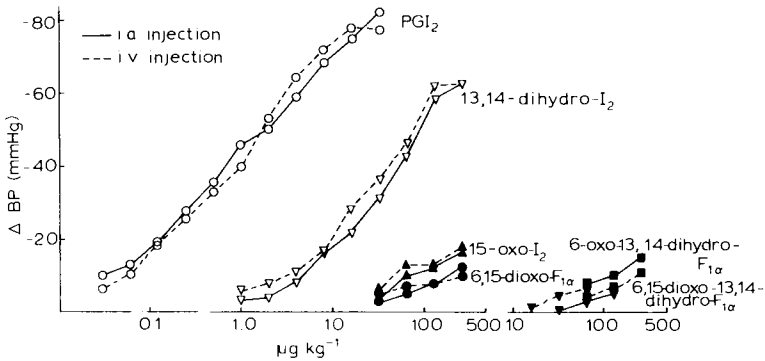


Figure 6.2. Effect of prostacyclin, and its metabolites on systemic arterial blood pressure (BP) in the anaesthetized rat following intravenous or intra-arterial bolus injection. Results, shown as the mean value for clarity, are from four experiments (adapted from Ref. 48).

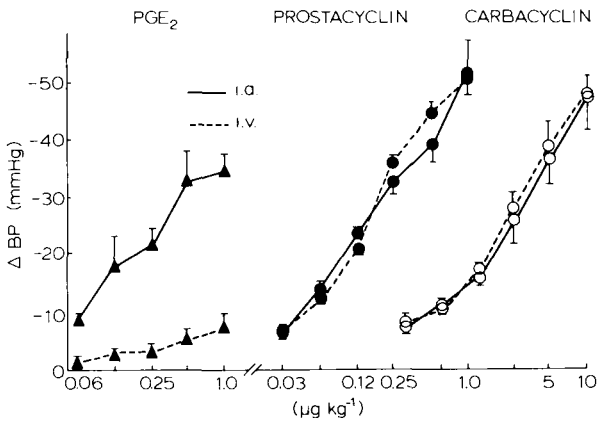


Figure 6.3. Effect of prostacyclin, carbacyclin and PGE_2 on systemic arterial blood pressure (BP) in the anaesthetized rat following intravenous or intra-arterial bolus injection. Results, shown as the mean ± S.E.M., are from four experiments (adapted from Ref. 32).

enzymes [47], all showed a considerable reduction in anti-aggregating activity *in vitro* [48], with 13,14-dihydro-PGI_2 [123] and 6-keto-$PGF_{1\alpha}$ being the most potent (*Table 6.3*). Similarly, these metabolites had reduced cardiovascular activity *in vivo* (*Figure 6.2*). Like prostacyclin, these metabolites all showed comparable vasodepressor activity when administered via the intravenous or intra-arterial route (*Figure 6.2*), indicating minimal pulmonary metabolism. Prostacyclin, like prostaglandin E_2 (PGE_2), which is readily metabolized by the lung *in vivo* (*Figure 6.3*), is a substrate for the isolated 15-PGDH enzyme, but, unlike PGE_2, is not a substrate for the pulmonary-uptake system required for metabolism by this enzyme [49], although it is metabolized readily in other organs such as kidney, liver and perhaps vascular tissue. In our studies [48], the putative prostacyclin metabolite, 6-keto-PGE_1, was 20-fold less active than prostacyclin in inhibiting human platelet aggregation itself, whereas 6-hydroxy-PGE_1 was only weakly active (*Table 6.3*).

The 13,14-dehydroprostacyclin methyl ester was reported to be more stable than prostacyclin (free acid), its stability over a 24 h study period being attributed to its methyl ester grouping [46]. Studies on its anti-aggregating activity in human PRP indicated it to have 0.1-times the activity of prostacyclin, both in inhibiting aggregation (*Table 6.4*) and the platelet release reaction, while this analogue also elevated platelet cyclic AMP levels [50]. Studies *in vivo* indicated that this 13,14-dehydro analogue was a vasodilator in the feline intestinal vascular bed, where it reduced perfusion pressure, as well as inducing

a prolonged reduction in perfusion pressure in the pulmonary vascular bed [51]. In a study in which its vasoactive properties on the dog coronary circulation were compared with those of prostacyclin, this analogue was some 3-times less active in increasing coronary sinus blood flow [52].

Table 6.4. ANTI-AGGREGATING POTENCY OF UNSTABLE OR MORE STABLE PROSTACYCLIN DERIVATIVES IN HUMAN PLATELET-RICH PLASMA IN COMPARISON WITH THAT OF PROSTACYCLIN

	Relative potency	*Reference*
PGI_2	1	
13,14-Dehydro-PGI_2 methyl ester	0.1	50
12-Fluoro-PGI_2	1	56
14-Fluoro-PGI_2	1	57
10,10-Difluoro-13,14-dehydro-PGI_2	0.1	59
13-Oxo-PGI_2	0.1	53
13-Thio-PGI_2	0.04	53
16-Cyclohexyl-PGI_2	0.1	60

In a series of presumably unstable 13-substituted prostacyclins, 13-oxo- and 13-thioprostacyclins had 0.1- and 0.04-times the activity of the parent as inhibitors of platelet aggregation [35].

The unstable higher homologue, 20-methylprostacyclin, was reported to inhibit the *in vitro* aggregation of platelets from rat and rabbit in doses comparable with those of prostacyclin [37,54]. However, in our studies on human platelet aggregation *in vitro*, 20-methylprostacyclin was 0.4-times as active, whereas the 20-ethyl derivative had a potency comparable with that of prostacyclin (*Table 6.2*). 20-Methylprostacyclin, like its parent, reduced aspecifically induced bronchoconstriction in asthmatic patients when given by inhalation in doses causing changes in heart rate [55].

The 13,14-dehydro derivative of 20-methylprostacyclin was considered to be some 3-times more active than prostacyclin in inhibiting rabbit platelet aggregation [37], whereas the comparable 15-epi derivative had reduced activity, as found with prostacyclin in the present studies on human PRP (*Table 6.2*). Substitution of a 13,14-dehydro grouping did not, however, enhance the anti-aggregating activity of 6-keto-$PGF_{1\alpha}$ (*Table 6.3*).

In a study on several 12-fluoroprostacyclins [56], the entantiomerically-pure (15*S*) epimer was as active as prostacyclin as an inhibitor of ADP-human PRP aggregation, this being suprising since it was tested as methyl ester. Although

its hydrolysis to the free acid would occur in plasma, this would not be expected to occur to such an extent in vascular strips of cat coronary artery *in vitro*, where it was also considered as active as prostacyclin [56]. The racemic mixture was 0.13-times as active, while the racemic (15*R*) mixture was 0.03-times active as prostacyclin in inhibiting aggregation in human PRP. Likewise, (±)-14-fluoroprostacyclin as the sodium salt was described as equipotent as prostacyclin on both assay systems, but there was no information on the assay conditions [57].

The electron-withdrawing properties of substituted fluorine atoms on the enol-ether system, such as 10,10-difluoro-13,14-dehydroprostacyclin resulted in a more stable molecule, with a half-life of 24 h when incubated at 37°C at pH 7.4 in a Krebs' solution [58]. A point to be raised here is that the authors refer to the biological half-life of the compound, since they used a bioassay to determine the decrease in its activity on storage. This could create confusion, since what was actually determined was the chemical half-life, but using a biological assay. The biological half-life should more appropriately refer to the pharmacokinetic and pharmacological parameters *in vivo*, and indeed, the authors show that the duration of haemodynamic actions in the anaesthetized dog was only comparable with that of prostacyclin, despite its increased chemical stability. The compound had 0.1-times the activity of prostacyclin (*Table 6.4*) as an inhibitor of human platelet aggregation [59]. Like prostacyclin, 10,10-difluoro-13,14-dehydroprostacyclin elevated platelet cyclic AMP levels, while this and the anti-aggregatory effect were reduced by concurrent incubation with SQ 22536, an inhibitor of adenylate cyclase [59]. Studies *in vitro* indicated that the analogue was 3-fold more active than prostacyclin in relaxing helical strips of canine mesenteric arteries and bovine coronary arteries, while it was equipotent in reducing BP in the anaesthetized dog following bolus intravenous injection [58].

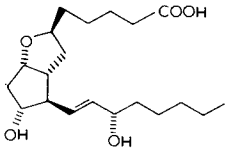
In a study on the vasodilator and anti-aggregating activity of a series of ω-side-chain modified analogues, replacement of the *n*-pentyl moiety with a cyclohexyl or (3-thionyl)-ethyl grouping reduced activity 10- and 100-fold, respectively, while a (3-thienyl)-oxymethyl substituent gave activity close to the parent [23,60].

An interesting approach to increase the chemical stability of the prostacyclin in solution has utilized β-cyclodextrine complexes of prostacyclin ethyl ester [61].

STABLE PROSTACYCLIN ANALOGUES

PGI_1 DERIVATIVES

One of the first chemically stable series of analogues to be described was the 5,6-dihydro analogues of prostacyclin [62–65,124]. The 6β-analogue (3)



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inhibited platelet aggregation in human PRP, being 0.003-times as active as prostacyclin (*Table 6.5*). As with prostacyclin, its vasodepressor activity was similar when administered by either intravenous or intra-arterial route in both rat and rabbit [48]. The epimer, 6α-PGI_1 was less active on the cardiovascu

Table 6.5. INHIBITION OF ADP-INDUCED HUMAN PLATELET AGGREGATION BY 5,6-DIHYDRO ANALOGUES OF PROSTACYCLIN *IN VITRO*

Results, given as the IC_{50} value (concentration causing 50% inhibition) following 1 min incubation at 37 °C in PRP and the relative potency to prostacyclin, are the mean ± S.E.M. from at least three experiments.

	IC_{50} (ng ml^{-1})	Relative potency
PGI_2	0.4 ± 0.1	1
6,7-Didehydro-PGI_1	12 ± 3	0.33
6β-PGI_1	116 ± 20	0.003
6α-PGI_1	350 ± 30	0.001
2-Nor-6β-PGI_1	> 2000	< 0.0002
2,3-Didehydro-6β-PGI_1	87 ± 10	0.0046
(11*S*)-6β-PGI_1	> 2000	< 0.0002
20-Methyl-6β-PGI_1	155 ± 5	0.003
20-*n*-Pentyl-6β-PGI_1	97 ± 19	0.004
(15*R*)-6β-PGI_1	> 2000	< 0.0002
15-Keto-6β-PGI_1	> 2000	< 0.0002
13,14-Dihydro-6β-PGI_1	860 ± 50	0.0005
(15*S*)-Methyl-6β-PGI_1	> 2000	< 0.0002
16,16-Dimethyl-6β-PGI_1	> 2000	< 0.0002
16,16-Difluoro-6β-PGI_1	94 ± 18	0.004

lar parameters and platelet aggregation (*Table 6.5*). As with prostacyclin, 6β-PGI_1 showed comparable anti-aggregating activity in human PRP, albumen-free suspensions of washed platelets and whole blood, indicating minimal non-reversible binding to plasma protein and other blood components [31].

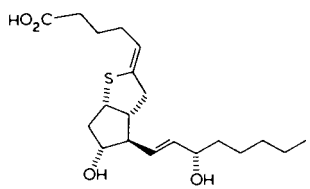
To study the chemical stability of 6β-PGI_1, aqueous solutions (100–500 μg ml^{-1}) were dissolved in sterile isotonic saline (0.9%(w/v); pH 7) and stored at 22°C in stoppered glass vials. At regular intervals, the potency of the stored solutions was tested for the ability to inhibit ADP-induced platelet aggregation in human PRP and compared with that of the freshly dissolved compound. There was no loss in biological activity in samples stored for 7 days at room temperature.

The biological activity of 5,6-dihydroprostacyclin analogues have also been described by other workers. The anti-aggregatory activity of both the 6α- and 6β-epimers on platelets has been reported by two other groups [64,66], while the analogue termed 'exo-PGI_1' has been shown to be 0.006-times as active as prostacyclin as an inhibitor of human platelet aggregation [67]. In a study on the guinea-pig isolated heart and bovine coronary artery strips, a divergent profile of activity of both α- and β-epimers of PGI_1 with prostacyclin was observed [68]. Thus, although these compounds have many of the properties of the parent compound, these 5,6-dihydro analogues cannot be considered as close mimics of prostacyclin at all prostacyclin-sensitive sites.

The 13,14-didehydro derivatives of 20-methyl-PGI_1 were found to stimulate cyclic AMP levels in human platelets [69], as well as in cultured endothelial and smooth-muscle cells derived from bovine arteries [70]. The synthesis of a further stable derivative, 4,5,6,7-tetradehydro-PGI_1 has been reported, having a calculated potency of approximately 0.17-times that of prostacyclin as an inhibitor of rabbit platelet aggregation, although experimental details were not stated [71].

THIAPROSTACYCLINS

The (5*Z*)-6,9-thiaprostacyclin, in which the ring oxygen atom of prostacyclin is substituted (4), was reported to be an active, more stable, prostacyclin



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derivative, [72], although this compound showed biological properties divergent from prostacyclin in that it constricted the cat isolated coronary artery. As an inhibitor of rabbit platelet aggregation *in vitro*, 6,9-thiaprostacyclin was 0.04-times less active than prostacyclin [73], similar to its relative potency of 0.02-times prostacyclin in the present studies in human PRP (*Table 6.6*). The (5*E*) isomer of 6,9-thiaprostacyclin was reported to be 'relatively inactive' in inhibiting platelet aggregation [72]. Following intravenous infusion in the anaesthetized cat, (5*Z*)-6,9-thiaprostacyclin lowered systemic BP and elevated superior mesenteric artery blood flow while inhibiting platelet aggregation *ex vivo* [74]. In the canine femoral circulation, it was 10-times less active as a vasodilator than prostacyclin [75]. These latter workers reported its half-life in neutral solution (determined by biological activity) to be 7 h. This analogue was 10–20-times less active than prostacyclin *in vivo* in causing disaggregation of platelets from cat blood perfused collagen strips, but this effect was not

Table 6.6. INHIBITION OF ADP-INDUCED HUMAN PLATELET AGGREGATION BY PROSTACYCLIN ANALOGUES *IN VITRO*

Results, given as the IC_{50} value (concentration causing 50% inhibition) following 1 min incubation at 37 °C in PRP and the potency relative to prostacyclin, are the mean ± S.E.M. from at least three experiments.

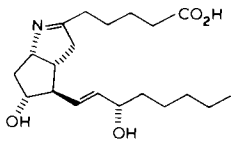
	IC_{50} (ng ml^{-1})	Relative potency
Prostacyclin	0.4 ± 0.1	1
6,9 α-Thiaprostacyclin	19 ± 1	0.02
16,16-Dimethylthiaprostacyclin	825 ± 72	0.0005
9-Deoxy-9α-5-nitrilo-PGF_1	> 2000	< 0.0002
9-Deoxy-9α-6-nitrilo-PGF_1	13 ± 3	0.03
(15*S*)-15-Methylnitrilo-PGF_1	960 ± 50	0.0004
9-Deoxy-6, 9α-imino-PGF_1	> 2000	< 0.0002
5α-5,9α-Epoxy-PGF_1	39 ± 8	0.01
5β-5,9α-Epoxy-PGF_1	310 ± 81	0.01
Δ^2-5α,9α-Epoxy-PGF_1	8 ± 1	0.05
Δ^2-5β,9α-Epoxy-PGF_1	100 ± 25	0.004
5α-13,14-Dihydro-5,9α-Epoxy-PGF_1	92 ± 13	0.004
Δ^2-5α-13,14-Dihydro-5,9α-epoxy-PGF_1	33 ± 5	0.01
Benzindine analogue	7 ± 2	0.06
20-Methylbenzindine	6 ± 0.4	0.07
16,16-Difluorobenzindine	2 ± 0.3	0.2
13,14-Dihydrobenzindine	11 ± 3	0.03

long-lasting and the compound possessed thromboxane A_2-like constrictor activity on isolated vascular tissue [73].

A series of 13-thia-substituted prostacyclins has been recently reported, with the 13,14-dihydro-13-thiaprostacyclin being 0.04-times as active as prostacyclin on human platelet aggregation, but whether these compounds were more chemically stable than the parent was not reported [53].

NITROGEN-CONTAINING ANALOGUES

Substitution of the ring oxygen moiety of prostacyclin by nitrogen has produced stable analogues with effective anti-aggregating potency. The nitrilo-analogue, 9-deoxy-9α-6-nitrilo-PGF_1 (5), was considered to be of polarity comparable with that of prostacyclin with the sp_2 hybridization of C-6 allowing adoption of confirmations equivalent to those of the parent [76]. In our studies, this



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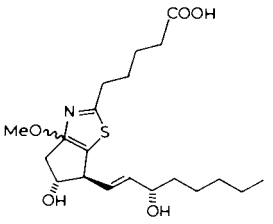
analogue was 30-fold less active than prostacyclin as an inhibitor of human platelet aggregation *in vitro* (*Table 6.6*) and likewise has reduced systemic vasodepressor activity *in vivo* (*Table 6.11*). This compound, like prostacyclin, was a pulmonary vasodilator in the lamb, under both hypoxic and normoxic conditions, and reduced the pulmonary-systemic resistance ratio during hypoxia, but was some 5–17-fold less active [77]. The 9-deoxy-6,9α-imino and the 9α-5-nitrilo derivatives were essentially devoid of anti-aggregating activity, while the 15-methylnitrilo analogue had reduced activity *in vitro* (*Table 6.6*). Further substitution, such as in the 16-cyclopentyl derivative, 16,18-ethano, or the 17(*S*)-methyl derivatives of the nitrilo analogue, produced compounds which showed potent anti-aggregating activity *in vitro* with a calculated activity 3.5- and 8-times less than that of prostacyclin [78].

In another series of analogues, the tetrahydrofuran ring of prostacyclin was replaced by a thiazole nucleus [79]. Of this series, the methoxy derivative (6) was the more potent, with a calculated potency of 0.003-times prostacyclin as an inhibitor of platelet aggregation *in vitro*.

Further substitution has led to a series of 5-oxo-, 5-imino- and 5-thia-9,6-nitrilo-PGF_1 derivatives [23], of which the β-thiaiminoprostacyclin



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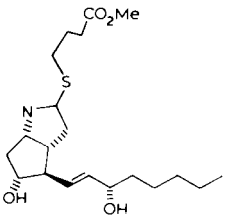

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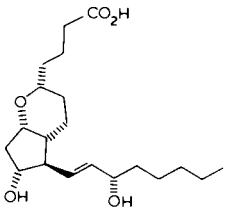
methyl ester was shown to have anti-aggregating actions both *in vitro* and *in vivo* in the rabbit [80]. Compound (7) known as Hoe 892, was 0.01-times as active as prostacyclin as an inhibitor of ADP-induced aggregation in rabbit PRP, but was apparently more active against collagen or arachidonate-induced aggregation [80]. The compound was orally effective in the rabbit as an inhibitor of platelet aggregation *ex vivo*, and induced cardiovascular changes in rats and dogs following bolus intravenous administration [80].

BENZINDENE, INTERPHENYLENE AND 5,9-EPOXY DERIVATIVES

A further modification of the chemically-stable dihydroprostacyclins was the expansion of the ring to give the family of 9-deoxy-5,9α-epoxy-PGF_1 derivatives



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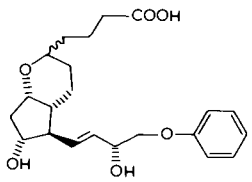
[63,81,125]. In our studies, the 5α analogue (8) was 100-fold less active than prostacyclin *in vitro* as an inhibitor of human platelet aggregation, with the 5β analogue being considerably less active (*Table 6.6*). The Δ^2 derivatives showed enhanced activity, being more potent than the corresponding 13,14-dihydro-Δ^2 analogue (*Table 6.6*). Substitution of a 16-phenoxy grouping substantially reduced anti-aggregating activity, with the 5β-16-phenoxy derivative (9) actually inducing aggregation at a concentration of 2 μg ml^{-1} [82].



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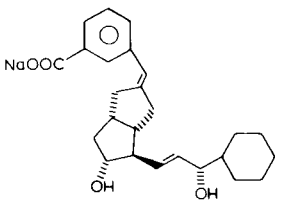
Replacement of the first four carbon atoms of prostacyclin with a carboxyphenylene residue was undertaken in an attempt to stabilize the labile enol ether bond [83]. Further modifications, by replacement of the terminal *n*-pentyl group in the ω-chain by a cyclohexyl grouping gave a relatively stable prostacyclin analogue which inhibited human platelet aggregation. Comparable substitution in the carbacyclin derivative yielded an interphenylene analogue (10) only 4-times less active as a platelet inhibitor than the corresponding oxo-cyclic analogue. As with prostacyclin and carbacyclin, the *Z*-isomers of the oxo-cyclic series and the *E*-isomers of the carbocyclic series, respectively, were the more active [83]. Infusion of the carbocyclic analogue (10) in the conscious rat lowered blood pressure and elevated plasma renin activity, while reducing diuresis [84].

It had previously been known that interphenylene prostaglandins had prostacyclin-like activities and it was proposed that by making the interphenylene derivative of a more conformationally rigid structure by forming a cyclopen-



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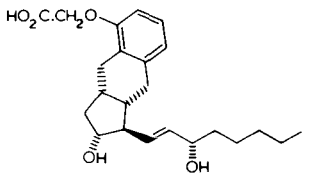

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tane ring, the prostacyclin-like biological profile could be enhanced [85]. Such an approach has led to the highly active tricyclic series of stable prostacyclin analogues, the benzindenes [86]. In our studies, the parent benzindene (11) was a potent inhibitor of human platelet aggregation, being 12-times less active than prostacyclin (*Table 6.6.*). The 20-methyl benzindene derivative had anti-platelet activity comparable to that of the 16,16-difluoro and 13,14-

dihydro deravatives (*Table 6.6.*). The *para*-benzindene analogue was virtually inactive, suggesting the stringent conformational requirements of this molecule for its binding to the platelet prostacyclin receptor.

CARBOPROSTACYCLINS

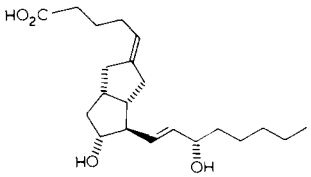
The synthesis and biological actions of stable carbocyclic analogues of prostacyclin, in which the enol-ether oxygen atom is replaced by a methylene group, have been described by several groups of workers [87–93]. Carbacyclin, or (5*E*)-6a-carbaprostaglandin I_2 (12) inhibited human platelet aggregation induced by a variety of agents including ADP, collagen and arachidonic acid, while also inhibiting aggregation in PRP obtained from rabbit, rat and dog [32,90–92]. In our studies, carbacyclin (12) was 0.02–0.03-times as potent as prostacyclin as an inhibitor of aggregation induced by ADP (*Table 6.7*),

Table 6.7. INHIBITION OF ADP-INDUCED HUMAN PLATELET AGGREGATION BY STABLE 6a-CARBOCYCLIC PROSTACYCLIN ANALOGUES *IN VITRO*

Results, given as the IC_{50} value (concentration causing 50% inhibition) following 1 min incubation at 37 °C in PRP and the relative potency to prostacyclin, are the mean ± S.E.M. from at least four experiments.

Carbocyclin	*IC_{50} (ng ml^{-1})*	*Relative potency*
(5*E*)-	11 ± 3	0.036
(5*Z*)-	700 ± 100	0.0006
(5*E*)- Methyl ester	140 ± 40	0.003
(5*E*)-(15*R*)-	1600	0.0003
(5*Z*)-(15*R*)-	> 2000	< 0.0002
(5*E*)-2-Nor-	> 2000	< 0.0002
(5*Z*)-2-Nor-	> 2000	< 0.0002
(5*E*)-19-Hydroxy-	443 ± 67	0.001
(5*E*)-16,16-Difluoro-	11 ± 1	0.04
(5*Z*)-16,16-Difluoro-	387 ± 49	0.001
2,2-Difluoro-	135 ± 48	0.003
15-Cyclohexyl	13 ± 1	0.03
(5*Z*,9*S*)-Ethynyl-	2.2 ± 0.3	0.18
9-Chloro-	1.3 ± 0.3	0.25
9-Cyano-	7.1 ± 0.3	0.06
9-Methyl-	87 ± 11	0.005
9-Pent-1-ynyl-	475 ± 175	0.0008
9-Ethyl-	1348 ± 439	0.0003
9-Carboxy-	> 2000	< 0.0002

collagen and arachidonic acid [32]. As with prostacyclin, the anti-aggregating activity of carbacyclin was enhanced by preincubation with the phosphodiesterase inhibitor, theophylline [32,92], supporting stimulation of cyclic AMP as its mechanism of inhibitory action. Direct studies have shown an elevation of platelet cyclic AMP following incubation with the analogue [91].



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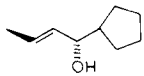

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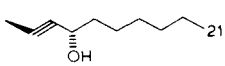

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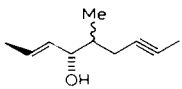

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Carbacyclin was a potent inhibitor of *ex vivo* platelet aggregation when infused intravenously in the rabbit (*Figure 6.4*) and dog, being one-tenth as active as prostacyclin [32]. Likewise, this analogue was effective *in vivo* in reducing thrombus formation in dog coronary arteries [40]. Studies with carbacyclin in anaesthetized baboons have likewise indicated inhibition of platelet aggregation *ex vivo* following intravenous or intragastric administration [94]. In more recent studies in human volunteers, intravenous infusion of carbacyclin (20–80 ng kg^{-1} min^{-1}) reduced *ex vivo* platelet aggregation at doses

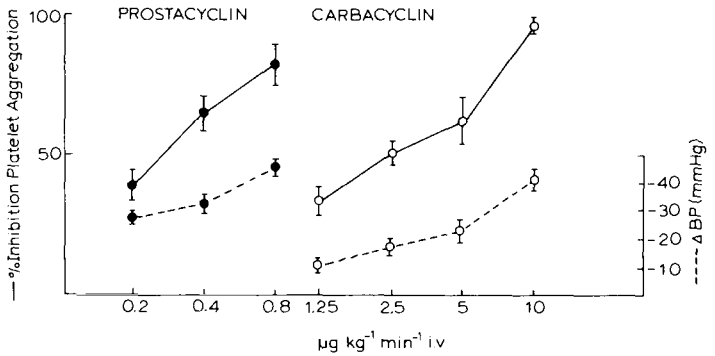


Figure 6.4. Inhibition of ADP-induced platelet aggregation ex vivo *and fall in systemic arterial blood pressure (BP) by intravenous infusion of prostacyclin in anaesthetized rabbits. Results, expressed as percentage inhibition of platelet aggregation compared to the initial controls, and the change in BP, are shown as the mean ± S.E.M. of four experiments for each value [32].*

which did not produce cardiovascular changes, whereas with oral administration of carbacyclin (25 mg kg^{-1}) inhibition of platelet aggregation was accompanied by headache, facial flushing and cardiovascular changes [95].

In our studies in human PRP, (5*Z*)-carbacyclin was less active than the (5*E*) analogue, the latter being isosteric with (1), naturally occurring prostacyclin ((5*Z*)-PGI_2). As expected, the (15*R*) epimers had minimal activity, as did the 2-nor derivatives, while the 16,16-difluoro-substituted carbacyclins [126] retained the *in vitro* activity of their parent carbacyclins (*Table 6.7*). Substitution in the 9-position [122] can also alter anti-aggregating potency, with the (5*Z*) isomers being more active, and the 9β-ethynyl, 9-chloro and 9-cyano groupings enhancing activity and 9β-methyl, 9-pent-1-ynyl, 9-carboxy and

Table 6.8. INHIBITION OF PLATELET AGGREGATION IN PRP FROM VARIOUS SPECIES BY CARBOCYCLIC PROSTACYCLIN ANALOGUES, AND THEIR RELATIVE POTENCY TO PROSTACYCLIN

Compound	*Species*	*Potency ratio*	*Reference*
(5*E*)-Carbacyclin	human	0.03	32
13,14-Didehydro-	rabbit	0.09	45
20-Methyldidehydro-	rabbit	0.11	45
15-Cyclopentyl-	human	0.11	96
	baboon	0.31	97
16-Methyl-18,19-didehydro-	human	1	98

9-ethyl groupings decreasing activity compared with that of carbacyclin itself (*Table 6.7*).

Studies using derivatives of carbacyclin have been reported, with the 15-cyclopentyl-ω-pentanorcarbacyclin derivative (13), being 10-fold less active than prostacyclin as an inhibitor of human platelet aggregation *in vitro* (*Table 6.8*). Further, the analogue (13) could inhibit platelet aggregation *ex vivo* following intravenous infusion or oral administration in the baboon and exhibited vasodepressor actions following bolus intravenous administration [96]. In further studies in human volunteers, 15-cyclopentylcarbacyclin (13; ONO 41483) infused intravenously in the maximum tolerated dose (2.5 ng kg^{-1} min^{-1}) caused a 27% inhibition of platelet aggregation *ex vivo*. Higher doses, both by the intravenous and by the oral route, which produced a more substantial inhibition of platelet aggregation, induced side-effects including flushing of the face and limbs, headache and phlebitis [97], similar to those observed with high doses of prostacyclin [36].

Further chemically stable analogues, based on the carbacyclin structure have been reported. Thus the 16-methyl-18,19-didehydro derivative (15) has been shown to have potent anti-aggregating properties, inhibiting human platelet aggregation *in vitro* induced by a variety of agents [98] over a concentration range similar to that of prostacyclin (*Table 6.8*). This analogue (ZK 36374 or iloprost) also induced *in vitro* disaggregation of platelets in cat whole blood, such platelet aggregates being formed *in vivo* following surgery, while intravenous infusion reversed the fall in platelet count associated with a 5 h period of myocardial ischaemia following coronary artery ligation in the cat [99]. Other studies in the rat have shown this derivative (15) to inhibit *in vitro* platelet aggregation in rat PRP, having 0.3-times the activity of prostacyclin. This potency is similar to that in the rat *in vivo* in prolonging bleeding time following intravenous infusion using a novel technique in which the time of occlusion of an arteriovenous shunt constructed of polyethylene tubing is determined [42]. Potent vascular relaxation of bovine coronary artery strips *in vitro* and vasodepression in cat and rat have also been observed with this carbacyclin derivative [42,99].

A series of 13,14-didehydrocarbacyclin analogues has also been described following the observations that the (5*E*)-13,14-didehydro analogue of PGI_1 with the extended 20 methyl-ω-chain was some 30-times less active than prostacyclin [100]. Compound (14), like its 20-carbon homologue, inhibited rabbit platelet aggregation *in vitro*, while studies *in vivo* in the guinea-pig using pulmonary accumulation of indium-labelled platelets following ADP-infusion as an index of aggregation, showed anti-aggregating activity following bolus injection [44].

METABOLIC STABILITY

METABOLISM OF PROSTACYCLIN AND ITS ANALOGUES

Although the chemical half-life of prostacyclin at physiological temperatures and pH is short, its anti-platelet activity, and particularly its cardiovascular activity *in vivo* (*Table 6.9*), is often even more short-lived [32]. This suggests

Table 6.9. HYPOTENSIVE POTENCY AND DURATION OF CARDIOVASCULAR ACTION OF PROSTACYCLIN AND ITS DERIVATIVES IN THE ANAESTHETIZED RAT

Dose-response relationships for the fall in mean BP with the compounds following bolus intravenous injection were constructed and compared with those obtained with prostacyclin in the same animal, and a potency ratio calculated. An index of the duration of the hypotensive effect for each compound was obtained by measuring time from the peak response to when it had recovered to half its peak response ($T_{1/2}$ min) for a standard fall in BP (Δ 30 mmHg).

	Hypotensive potency	$T_{1/2}$ *(min)*
Prostacyclin	1	0.6
(5*E*)-PGI_2	0.02	1.0
15-Methyl-PGI_2	0.03	0.9
16,16-Dimethyl-PGI_2	0.06	0.7
20-Methyl-PGI_2	1.0	0.5
20-Ethyl-PGI_2	1.7	0.6
19-Hydroxy-PGI_2	0.08	0.9
(15*R*)-PGI_2	0.05	0.4

that the pharmacological effects *in vivo* are limited by biological inactivation rather than solely by chemical breakdown. Unlike the more classical prostaglandins of the E and F series, prostacyclin has comparable vasodepressor actions when injected by the intra-aortic or intravenous route in rats, rabbits, or dogs (*Figure 6.3*), suggesting that pulmonary degradation is not of primary importance in prostacyclin inactivation [32]. The failure of prostacyclin to be metabolized during passage through the lung indicates that it is not a substrate for the pulmonary-uptake system required for metabolism by the enzyme, 15-hydroxyprostaglandin dehydrogenase (15-PGDH) in the intact lung [46], since prostacyclin is a substrate for the isolated enzyme [101]. In contrast, the breakdown product 6-keto-$PGF_{1\alpha}$ is not a good substrate for this enzyme.

Prostacyclin is metabolized during passage through the peripheral circulation, liver and kidney, although the rate and nature of the metabolic processes

are complex [47,102]. Studies on the composition of the urinary and bilary metabolites of prostacyclin following its infusion in rats indicate that it can undergo the metabolic transformations described for the more classical prostaglandins. Thus, products resulting from 15-dehydrogenation, Δ^{13}-reduction, β-oxidation, 19- and 20-hydroxylation and oxidation have been identified [47,103]. Likewise, 6-keto-$PGF_{1\alpha}$ resulting from chemical breakdown of prostacyclin *in vivo* may further be metabolized, as shown in studies in man [104,105], while studies in dogs have indicated a rapid elimination of both prostacyclin or 6-keto-$PGF_{1\alpha}$ from the plasma following intravenous infusion [106].

The metabolites of prostacyclin and 6-keto-$PGF_{1\alpha}$ in general have minimal biological activity [46], either as inhibitors of platelet aggregation (*Table 6.3*) or as vasodepressors (*Figure 6.2*). The 15-hydroxy grouping is clearly essential for activity, whereas the 13,14-dihydroprostacyclin retains some biological activity.

Although chemically stable, analogues such as 6β-PGI_1 and carbacyclin have only short-lived biological activity *in vivo*, as determined by cardiovascular actions and inhibition of platelet aggregation *ex vivo*, suggesting that like prostacyclin, these compounds are rapidly inactivated [32]. Further, like prostacyclin, both 6β-PGI_1 and carbacyclin have comparable vasodepressor activity when administered by the intravenous or intra-arterial route, indicating minimal pulmonory inactivation (*Figure 6.3*). Since carbacyclin is a good substrate for the purified 15-hydroxy-prostaglandin dehydrogenase derived from monkey lung [107], this again suggests that these analogues fail to gain access to the enzyme in the intact lung. Like prostacyclin, these analogues will presumably undergo hepatic metabolism.

METABOLIC PROTECTION

The prevention of enzymatic inactivation of prostacyclin analogues *in vivo* has been attempted by substitution in both β- and ω-chains. In other prostaglandin series such as the E-series, protection of the 15-hydroxy grouping by the substitution of a bulky moiety such as a 15-methyl or 16,16-dimethyl group has greatly prolonged and even enhanced the biological activity *in vivo* [108–110]. Furthermore, since the Δ^{13-14} reductase accepts 15-keto prostaglandins more readily, protection against the 15-hydroxy dehydrogenase should also reduce the potential for inactivation by this reductase. However, such 15-methyl and 16,16-dimethyl substitutions in the prostacyclin structure, which reduced *in vitro* platelet activity (*Table 6.2*), did not significantly enhance the duration of the biological activity *in vivo*, as indicated by the duration of the vasodepressor activity following bolus injection in the anaesthetized rat

(*Table 6.9*). Introduction of substituents which could prevent ω-oxidation, such as in the 20-methyl or 19-hydroxy derivatives, likewise did not prolong the duration of action. Furthermore, similar substitutions in chemically stable prostacyclin analogues did not produce compounds with significantly longer-lasting cardiovascular actions *in vivo* (*Table 6.10*).

Introduction of a 15-methyl group into 6β-PGI_1, in an attempt to prevent attack on the 15-hydroxy by the enzyme, 15-PGDH, did not augment the duration of submaximal vasodepressor actions *in vivo* (*Table 6.10*). Likewise,

Table 6.10. HYPOTENSIVE POTENCY AND DURATION OF CARDIOVASCULAR ACTIONS OF STABLE ANALOGUES OF PROSTACYCLIN IN THE ANAESTHETIZED RAT

Dose-response relationships for the fall in mean systemic arterial blood pressure (BP) with the analogues following bolus intravenous injection were constructed and compared with those obtained with prostacyclin in the same animal, and potency ratio calculated. An index of the duration of the hypotensive effect for each compound was obtained by measuring time from the peak response to when it had recovered to half its peak response ($T_{1/2}$ min) for a standard fall in BP (Δ 30 mmHg).

	Hypotensive potency	$T_{1/2}$ *(min)*
Prostacyclin	1	0.6
6β-PGI_1	0.09	0.6
(15*S*)-15-Methyl-	0.01	0.45
16,16-Dimethyl-	0.04	1.2
Δ^2-	0.03	1.4
Carbacyclin	0.03	0.6
2,2-Difluoro-	0.01	0.6
16,16-Difluoro-	0.31	0.45
15-Cyclohexyl-	0.05	0.45
19-Hydroxy-	0.03	0.45

introduction of a 16,16-dimethyl substituent, as well as Δ^2 [124] to prevent β-oxidation did not substantially increase the duration of cardiovascular action. In the carbacyclin series, introduction of a 15-cyclohexyl or 16,16-difluoro group failed to enhance the biological half-life *in vivo*, as did protection against β- or ω-oxidation by 2,2-difluoro [126] and 19-hydroxy substitution, respectively (*Table 6.10*). In studies on platelet aggregation *ex vivo* following intravenous infusion in the anaesthetized rabbit, those prostacyclin analogues with 15- and 16-methyl substituents, as well as the chemically stable analogues, did not show a substantial increase in the duration of anti-platelet activity [32].

Pharmacokinetic studies with the 16-methyl-18,19-didehydrocarbacyclin (14) following bolus intravenous injection in the rat showed a biphasic decline in plasma concentrations, with an initial half-life of 5 min [110]. Although the pharmacokinetics of prostacyclin under comparable conditions was not investigated, these results suggest that the analogue does not exhibit a biologically significant longer duration of activity than prostacyclin, despite protection of the 15-hydroxy grouping from enzymatic attack.

In a further series of carbocyclic analogues, the 15-hydroxy group was protected by modification of the ω-tail, such as by insertion of a 15-cyclohexyl grouping, while protection against β-oxidation was afforded by insertion of an *m*-carboxyphenylene residue (10). Despite such modifications, these prostacyclin analogues still exhibited only a short duration of action, with the hypotensive effects ranging from 0.25–1.2 min [83] as found with other 'protected' carbacyclin analogues (*Table 6.10*). Only with extremely high supramaximal dosage levels were any differences between the duration of biological action of the analogues detected, and thus such actions have no clinical or experimental application.

These findings with chemically stable prostacyclin analogues thus suggest a rapid elimination of the analogues, largely independent of the rate of metabolism of the analogues, is of prime importance in dictating plasma levels of active drug and hence the duration of cardiovascular and anti-aggregating activity *in vivo*. It would thus appear that until the pharmacokinetic parameters of these analogues are fully elucidated and some way of controlling the distribution of the analogue from the blood to the various tissue compartment is developed, chemically and metabolically stable prostacyclin analogues with a long duration of action will elude identification.

SELECTIVITY OF ACTION

SELECTIVITY RATIO

A preliminary indication of the separation between platelet anti-aggregating actions and cardiovascular actions within a particular prostacyclin analogue series can be gained from consideration of the selectivity ratio, shown in *Table 6.11*. Studies on the ability of such analogues to inhibit human platelet aggregation *in vitro* and to lower BP in the anaesthetized rat can be employed as primary screens for biological activity of analogues. Thus, the ratio of the relative potency of the analogue to prostacyclin as a vasodepressor *in vivo* and as a platelet inhibitor *in vitro* can readily be obtained. Using this ratio, it can be seen that carbacyclin (12) has a selectivity ratio close to that of prostacyclin, whereas the nitrilo (5) and 5-9-epoxy-F_1 (8) analogues had higher ratios. This

Table 6.11. RELATIVE POTENCY AND SELECTIVITY RATIO OF PROSTACYCLIN AND ITS ANALOGUES

Relative potency and selectivity of prostacyclin analogues on ADP-induced human platelet aggregation *in vitro* and rat systemic arterial blood pressure *in vivo* (A) and on ADP-induced rabbit aggregation *in vitro* and rabbit systemic arterial blood pressure (B). Results are shown as the mean values from 3–6 experiments for each.

Compound	*Relative potency*		*Selectivity ratio*
	Platelet inhibition	*Blood pressure*	*BP/aggreg.*
A	*Human*	*Rat*	
PGI_2 (1)	1	1	1
Carbacyclin (12)	0.03	0.05	1.5
Nitrilo (5)	0.01	0.04	4.0
5,9-Epoxy-F_1 (8)	0.007	0.10	14.2
B	*Rabbit*	*Rabbit*	
PGI_2 (1)	1	1	1
Carbacyclin (12)	0.036	0.046	1.1
Nitrilo (5)	0.037	0.08	2.4
5,9-Epoxy-F_1 (8)	0.008	0.05	5.6

Table 6.12. RELATIVE POTENCY AND SELECTIVITY OF PROSTACYCLIN ANALOGUES AND PGE_1, ON ADP-INDUCED HUMAN PLATELET AGGREGATION *IN VITRO* AND RAT SYSTEMIC ARTERIAL BLOOD PRESSURE (BP) *IN VIVO*

Results are the mean values for 3–6 experiments for each. A ratio higher than 1 indicates a relatively greater action as a vasodepressor than anti-aggregating agent when compared with that of prostacyclin [32].

	Relative potency		*Selectivity ratio*
	Platelet inhibition Human	*Blood pressure Rat*	*BP/aggreg.*
Carbacyclin	0.03	0.05	1.5
(5*Z*)-Carbacyclin	0.005	0.0007	7
(15*R*)-Hydroxycarbacyclin	0.002	0.0002	10
6β-PGI_1	0.006	0.03	5
PGE_1	0.016	0.1	6.3

suggests that these latter analogues, like 6β-PGI_1 and PGE_1 (*Table 6.12*), are less selective than carbacyclin towards their platelet anti-aggregating actions. Thus, for an equivalent degree of platelet aggregation, one would predict a greater cardiovascular response with these latter analogues. A favourable ratio of activity for carbacyclin has also been reported by others [100], whereas the (5*Z*)- and (15*R*)-hydroxy derivatives of carbacyclin appeared less selective than carbacyclin itself (*Table 6.12*).

This selectivity ratio must be interpreted with caution, since it is derived from studies in two species, and from a comparison of *in vitro* and *in vivo* data. In order to investigate the interspecies variation, studies on both platelet aggregation *in vitro* and cardiovascular actions *in vivo* have been conducted in one further species, the rabbit. As can be seen in *Table 6.11*, the rank order of the selectivity ratio for prostacyclin and its stable analogues from platelet aggregation *in vitro* and cardiovascular studies *in vivo* in the rabbit is comparable with that derived from the human and rat studies, thus supporting the use of this ratio, at least for the study of prostacyclin analogues.

Another problem with this ratio is the comparison of *in vivo* and *in vitro* data. Thus modification of a structure to prevent metabolism for example, may reduce its *in vitro* activity compared with that of its parent, yet *in vivo* it may appear more potent as a consequence of reduced metabolism, giving a seemingly high ratio and apparent poor selectivity. Nowithstanding these comments, the selectivity ratio may help to identify those compounds with a potentially high or low selectivity towards either property and thus may be useful in preparing a shortlist of prostacyclin analogues for further evaluation of their actions *in vivo*.

SELECTIVITY *IN VIVO* AND STUDIES IN MAN

It is clear that the most valid determination of selectivity between cardiovascular and platelet anti-aggregating actions of prostacyclin analogues can be made from studies in which the compounds are administered *in vivo* and in which both parameters are measured simultaneously. For this purpose, the inhibition of platelet aggregation *ex vivo* and concurrent cardiovascular changes have been determined in anaesthetized rabbit, dog and monkey [32]. To enable a more rapid preparation of samples of PRP so that platelet function could readily and continually be assessed (especially during the administration of labile substances such as prostacyclin), a rapid-spin method for the centrifugation of blood samples was developed (*Figure 6.1*). Using this technique in the anaesthetized rabbit, the dose-response relationships for prostacyclin and its derivatives following intravenous infusion have been determined (*Table 6.13*).

The 16-phenyl, 16,16-dimethyl and the 6,9α-thia derivatives of prostacyclin were less potent than prostacyclin as inhibitors of *ex vivo* aggregation. In doses causing a comparable degree of platelet inhibition, these analogues had cardiovascular potency close to that of prostacyclin, whereas the 15-methyl derivative appeared less selective for the platelet action (*Table 6.13*).

Previous studies on the cardiovascular actions of the 15-hydroxy epimer of prostacyclin in the cat and rat had suggested that this compound had minimal

Table 6.13. EFFECT OF PROSTACYCLIN AND ITS DERIVATIVES ON *EX VIVO* PLATELET AGGREGATION AND SYSTEMIC ARTERIAL BLOOD-PRESSURE DURING INTRAVENOUS INFUSION IN ANAESTHETIZED RABBIT

Results, shown as mean ± S.E.M. of 3–6 experiments, are inhibition of ADP-induced aggregation *ex vivo* and concurrent changes in systemic arterial blood pressure (BP) during a 15 min period of intravenous infusion.

	μg kg^{-1} min^{-1}	*% inhibition*	*Δ BP (mmHg)*
Prostacyclin	0.2	40 ± 7	− 26 ± 3
	0.4	66 ± 8	− 30 ± 4
	0.8	83 ± 9	− 46 ± 3
(15*R*)-Hydroxy-PGI_2	8	0	− 14 ± 1
16,16-Dimethyl-PGI_2	2.5	25 ± 11	− 4 ± 3
	10	49 ± 7	−25 ± 7
16-Phenyl-PGI_2	1	45 ± 2	− 23 ± 4
	2	75 ± 3	−36 ± 4
Epithio-PGI_2	4	50 ± 4	− 16 ± 2
6β-PGI_1	10	17 ± 7	− 25 ± 3
	20	44 ± 13	− 28 ± 3
(15*S*)-Methyl-PGI_1	20	8 ± 5	− 26 ± 4
Nitrilo-PGI_2	1.25	14 ± 8	− 20 ± 3
	5	40 ± 15	− 36 ± 1
5,9-Epoxy-F_1	5	12 ± 3	− 26 ± 1
	20	49 ± 12	− 23 ± 4
Carbacyclin	2.5	49 ± 7	−18 ± 2
	5	52 ± 4	− 26 ± 4
	10	96 ± 3	− 40 ± 5

actions as a vasodilator, yet retained weak platelet activity [100]. However, the potency ratio to prostacyclin as vasodilator was not reported in that study and thus the true selectivity of this derivative cannot be assessed. In the present *in vivo* studies in the rabbit, the prostacyclin (15*R*)-hydroxy epimer caused a fall in BP during intravenous infusion in doses which had no inhibitory effect on platelet aggregation, indicating poor selectivity towards the platelet actions (*Table 6.13*).

Table 6.14. INHIBITION OF PLATELET AGGREGATION *EX VIVO* FOLLOWING INTRAVENOUS INFUSION OF PROSTACYCLIN AND ITS STABLE ANALOGUES IN THE ANAESTHETIZED RABBIT

Results are shown as the ID_{50} (dose causing 50% inhibition) value for inhibition of ADP-induced platelet aggregation and the concurrent fall in systemic arterial blood pressure (BP). Data from Ref. 32 and unpublished work.

Compound	*Platelet ID_{50} ($\mu g\ kg^{-1}\ min^{-1}$)*	*ΔBP (mmHg)*
Prostacyclin	0.3	−27
Carbacyclin	2.4	−14
6β-PGI_1	22	−32
Nitrilo	6	−40
5,9-Epoxy-F_1	20	−23

Of the stable prostacyclin analogues, the nitrilo analogue (5) and 6β-PGI_1 (3) also appeared less selective than prostacyclin, whereas the 5,9-epoxy-F_1 derivative (8) and carbacyclin (12) exhibited a greater selectivity towards the platelet anti-aggregating action (*Table 6.14*).

Further studies with carbacyclin in the anaesthetized dog suggested that inhibition of platelet aggregation *ex vivo* could be achieved following intravenous infusion in doses having minimal action on BP [32]. While carbacyclin appeared more selective than prostacyclin, this selectivity of action cannot be considered of great magnitude (*Table 6.15*). Cardiovascular studies in the anaesthetized baboon and determination of platelet inhibition *in vitro* with carbacyclin and prostacyclin also suggested that carbacyclin could offer some selectivity. Indeed, *ex vivo* studies in the baboon with carbacyclin indicated that near-maximal inhibition of platelet aggregation could be achieved in doses having minimal hypotensive action [94]. However, following oral administration of carbacyclin in a dose causing 70% inhibition of platelet aggregation, a rise in heart rate but no fall in BP was observed in the baboon [94]. In our

studies in the anaesthetized monkey, intravenous infusion of carbacyclin in intermediate doses inhibited platelet aggregation with minimal hypotensive actions (contrasting with the actions of prostacyclin), although at higher rates of infusion, carbacyclin did lower BP (*Table 6.15*).

Table 6.15. ACTIONS OF PROSTACYCLIN OR CARBACYCLIN ON *EX VIVO* PLATELET AGGREGATION AND SYSTEMIC ARTERIAL BLOOD PRESSURE (BP) IN THE ANAESTHETIZED RHESUS MONKEY AND DOG DURING INTRAVENOUS INFUSION

Platelet aggregation *ex vivo* was induced by ADP in doses causing submaximal aggregation (3 and 5 μM in monkey and dog, respectively) and maximal aggregation (10 and 20 μM, respectively). Results are mean ± S.E.M. from three or four experiments in either species during a 15 min period of intravenous infusion. Data from Ref. 32 and unpublished work.

Compound	*Dose*	*Platelet aggregation (% inhibition)*		*ΔBP (mmHg)*
		ADP sub-max	*ADP max*	
A. Monkey				
Prostacyclin	0.01	50 ± 10	17 ± 4	−6 ± 5
	0.02	85 ± 8	51 ± 7	−17 ± 6
	0.05	99 ± 1	82 ± 8	−36 ± 10
Carbacyclin	0.4	56 ± 7	22 ± 7	−1 ± 0.5
	0.8	81 ± 7	42 ± 10	−5 ± 3
	2.0	99 ± 1	88 ± 5	−30 ± 14
B. Dog				
Prostacyclin	0.01	40 ± 15	37 ± 15	−6 ± 2
	0.02	61 ± 4	54 ± 11	−11 ± 3
Carbacyclin	0.2	67 ± 24	64 ± 22	−4 ± 2
	0.4	86 ± 12	76 ± 21	−10 ± 5

In a preliminary evaluation in human volunteers, intravenous infusion of carbacyclin inhibited platelet aggregation *ex vivo* yet had no effect on the BP or heart rate [95]. Since conccurent studies with prostacyclin in these subjects were not undertaken, the extent of this selectivity of action in man cannot be assessed. After oral administration of carbacyclin (25 mg) in two subjects in a dose sufficient to cause approximately 68% inhibition of platelet aggregation

ex vivo (determined 30–90 min later), a marked rise in heart rate was observed, with the rapid onset of headache and flushing [95].

Selectivity of action may be more readily demonstrated in certain species and some models of platelet aggregation. This is exemplified by the studies with the hydantoin prostaglandin BW245C which, although not a prostacyclin analogue, does share its anti-aggregatory and cardiovascular properties [111]. In the anaesthetized rabbit, BW245C inhibited platelet aggregation *ex vivo* following intravenous infusion, yet, in contrast to prostacyclin, had minimal effect on BP. In the anaesthetized dog, however, profound changes in BP were observed with BW245C in anti-aggregating doses [111]. In the rhesus monkey during intravenous infusion, BW245C and prostacyclin had a comparable selectivity on platelet aggregation and BP, as also found in a study in human volunteers [112]. Such difference in the selectivity of different prostacyclin analogues *in vivo* between different species has not, however, been observed, although the potency ratio between the analogue and prostacyclin may vary (*Table 6.16*).

Studies with the substituted carbacyclin derivative (15) in the anaesthetized cat indicate that the decrease in peripheral platelet count following coronary ligation could be abolished in doses having no effect on BP [98]. However, comparative studies with this analogue and with prostacyclin on thrombus

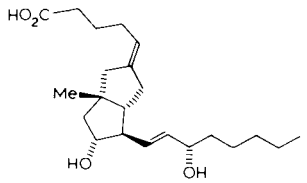
Table 6.16. INHIBITION OF ADP-INDUCED PLATELET AGGREGATION IN PLATELET-RICH PLASMA BY PROSTACYCLIN AND CARBACYCLIN *IN VITRO* FOLLOWING 1 MIN INCUBATION AND *EX VIVO* FOLLOWING INTRAVENOUS INFUSION IN VARIOUS SPECIES

Results are shown as ID_{50} (dose producing 50% inhibition) from at least three experiments for each value. Experimental data taken from Whittle et al. [32] while *ex vivo* human data are from [a] Ref. 36 and [b] Ref. 94.

	ID_{50} value			
	Prostacyclin		*Carbacyclin*	
	in vitro *(ng ml^{-1})*	ex vivo *(μg kg^{-1} min^{-1})*	in vitro *(ng ml^{-1})*	ex vivo *(μg kg^{-1} min^{-1})*
Human	0.3	0.008 [a]	11	0.2–0.8 [b]
Dog	0.7	0.015	20	0.15
Rabbit	2.0	0.28	25	2.4

formation in an extracorporeal circuit in the anaesthetized rat could not demonstrate any dissociation between the anti-aggregatory and vasodepressor actions [43]. It is not yet known whether selectivity of this analogue (15) can be demonstrated in studies of platelet aggregation *ex vivo* in various species including rabbit, dog and monkey, as well as in man.

Studies in the baboon with the 15-cyclopentylcarbacyclin derivative (13) has also suggested selectivity away from the cardiovascular actions of prostacyclin,



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with inhibition of platelet aggregation *ex vivo* being observed following oral or intravenous administration, with no change in BP or heart rate [96]. Although no direct study *ex vivo* was conducted, from comparison of the potency on human platelet aggregation *in vitro* and BP studies following bolus intravenous injection in the baboon, it was also suggested that this analogue was more selective than its parent, carbacyclin [96]. In subsequent studies in human volunteers with this analogue (13), changes in BP or heart rate were not detected in the doses used. However, rates of intravenous infusion of the analogue sufficient to cause 50% inhibition of platelet aggregation did induce facial flushing and vasodilation in the extremities, as also detected following oral administration [97].

In a double-blind placebo-controlled comparison of the effects of prostacyclin and the chemically stable analogue 9β-methylcarbacyclin (16; ciprostene) in healthy volunteers, both drugs exhibited a comparable profile of pharmacological activity [113]. Thus, during intravenous infusion, 9β-methylcarbacyclin (150–600 ng kg^{-1} min^{-1}) significantly displaced the dose-response curve for ADP-induced platelet aggregation *ex vivo* and was 0.01-times as active as prostacyclin. Elevation in heart rate and headache were observed only at the highest rate of infusion, as with prostacyclin, while no change in BP was observed with either compound in the dose range studied. A greater incidence of facial flushing was observed with prostacyclin at the lower dose range [113].

The mechanisms underlying peripheral vasodilation, as demonstrated by facial flushing or increased blood flow to the extremities in the absence of marked fall in BP, as seen with prostacyclin, 9β-methylcarbacyclin or other analogues, warrant further investigation and point to a potential therapeutic role in the treatment of peripheral vascular diseases.

FUTURE DEVELOPMENTS

From this survey of the pharmacological actions of prostacyclin and chemically synthesized analogues, it is clear that, although major advances have been made in the development of prostacyclin-like compounds, there are still important goals not yet achieved. From a consideration of the different classes of prostacyclin analogues and their subsequent structural variants, a strict requirement of the prostacyclin functional groups for the molecule to possess biological activity has emerged, with minor modifications leading to a loss of potency or alteration in pharmacological profile. Thus, even the 5,6-dihydro analogue, 6β-PGI_1 (3), is only a weak inhibitor of platelet aggregation compared with prostacyclin, although it does retain relatively more of the cardiovascular activity. Indeed, it appears from the data presented here that the binding or receptor sites on platelets for prostacyclin are more stringent in their structural requirements than those on the vasculature, with platelet activity being more readily lost than the cardiovascular activity.

The second generation of prostacyclins, exemplified by carbacyclin (12) and its derivatives (13)–(16), offer chemically stable but metabolically unstable analogues which exhibit a biological profile of activity comparable with that of prostacyclin. These analogues thus may offer pharmaceutical and clinical advantages over prostacyclin because of their ease of formulation, storage and use. Oral activity has also been achieved within this group of compounds, while pharmacological activity, especially on platelet function, has been demonstrated in human volunteers. Findings from the clinical use of these analogues for the therapy of various thromboembolic disorders are eagerly awaited.

Two major advances in drug design based on the prostacyclin molecule have yet to be realized. The biological half-life of these analogues, although in some cases of somewhat longer duration than that of prostacyclin, is still relatively short. Certain utilities may therefore require frequent or continuous administration, especially by the parenteral route. Following oral administration, longevity of the biological responses may simply reflect slow absorption, and

whether the rate of absorption is sufficiently constant or predictable for this to be of therapeutic value requires further pharmacokinetic data. Thus, the development of compounds resistant to metabolic inactivation or to elimination which provide long-lasting pharmacological effects *in vivo* will represent a third stage in the development of the prostacyclin analogue.

Despite some evidence for changes in selectivity away from the cardiovascular actions and towards the platelet action compared with prostacyclin, no analogue has yet been described that clearly separates both actions *in vivo* and thus a high selectivity for the platelet anti-aggregating property. The development of such highly selective analogues will ultimately depend on exploitation of any pharmacological differences between the receptor-binding sites on platelets and on vascular tissue. The binding characteristics of prostacyclin to human platelets have been reported by several groups [114–117], while the stable analogue 6β-PGI$_1$ has been used to investigate these binding sites and their coupling to the stimulation of platelet adenylate cyclase [118]. Receptor-binding studies using the carbacyclin derivative (15) on membranes isolated from coronary and aortic vascular tissue of ox and pig have been reported [119–120]. Stable prostacyclin analogues have also been used to investigate the stimulation of adenylate cyclase in cultured myocytes derived from rabbit aorta and mesenteric artery [121] and the elevation of cyclic AMP levels in cultured endothelial cells from bovine aorta [70]. However, there is as yet no detailed comparison between the platelet and vascular binding sites for prostacyclin and its analogues which would aid in the formal classification of these receptors, and the subsequent separation of these pharmacological activities.

The definitive separation of the platelet and cardiovascular properties of prostacyclin would undoubtedly be a major advance, representing a fourth generation of analogues, especially if combined with chemical stability, long duration of action and oral activity. In the absence of such fully selective analogues, the desirable degree of selectivity between such parameters is unknown, since both activities may be of importance in many of the proposed clinical utilities. The rational design of newer analogues will therefore depend on the clinical experience gained with prostacyclin itself. Furthermore, the current availability of stable analogues should accelerate the exploration of the clinical benefits of this class of compounds and provide impetus for the further development of such therapeutic agents. Thus, there remains a strong challenge to both synthetic chemists and pharmacologists to design and provide further compounds which will exploit fully the anti-thrombotic potential of such a potent endogenously occurring substance, prostacyclin.

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