Volume 31

PROGRESS IN MEDICINAL CHEMISTRY

Edited by

G.P. ELLIS and D.K. LUSCOMBE

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1994

ELSEVIER
AMSTERDAM · LONDON · NEW YORK · TOKYO

Elsevier Science BV
P.O. Box 211
1000 AE Amsterdam
The Netherlands

Library of Congress Cataloging in Publication Data:

Please refer to card number 62-27/2 for this series.

ISBN 0-444-81807-3
ISBN Series 0-7204-7400-0

This book is printed on acid-free paper

Printed in The Netherlands

Preface

We have pleasure in presenting nine reviews in this volume covering important advances in the chemistry and biology of a broad spectrum of medicinal topics. With the emergence of new insulin variants and some new synthetic hypoglycaemic drugs, a review of recent progress (Chapter 1) in the treatment of diabetes mellitus is timely.

Most enzymes present in the body play an essential biochemical role, but several of them also have an undesirable characteristic which requires selective suppression. Thus, the degrading effect of human leukocyte elastase on elastin (which provides elastic properties in several organs) requires selective inhibitors which are discussed in Chapter 2.

Inhibition of gastric H^+/K^+-ATPase (Chapter 4) provides an alternative treatment for gastric and duodenal ulcers to H_2-receptor antagonists (see Volume 20, Chapter 6). β-Lactamases severely limit the effeciency of some β-lactam antibiotics, but the discovery of inhibitors of these enzymes has restored the usefulness of several penicillins (Chapter 6).

A review of 16-membered macrolide antibiotics (Chapter 5) complements a survey of semi-synthetic erythromycins which appeared in Volume 30. Further work in this direction is expected to yield new antibiotics of greater efficiency and a lower level of side-effects. The antibacterial effects of silver have been known for a long time and are assessed in Chapter 7.

The azido group (with its well-known explosive tendency) has in recent years become a feature of several drugs (including AZT) which are showing considerable clinical potential; the medicinal chemical significance of this functional group is reviewed in Chapter 3. In contrast, the complex naturally occurring and potent vasoconstrictor, endothelin, presents medicinal researchers with a challenge to find a clinically useful inhibitor (Chapter 8). Finally, potassium channel activation is a relatively new type of pharmacological mechanism; its significance and potential is reviewed in Chapter 9.

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July 1993

G.P. Ellis
D.K. Luscombe

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1 New Hypoglycaemic Agents

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INTRODUCTION

Diabetes mellitus is a disease characterized by excess glucose in the plasma and by various other metabolic abnormalities, including dyslipidaemia. In the United States, between 6 and 12 million people are believed to be affected, among which the majority suffer from non-insulin dependent diabetes mellitus (NIDDM or type II) and the rest (500,000) from insulin dependent diabetes mellitus (IDDM or type I) [1,2]. The development of NIDDM has been linked to both genetic factors (the prevalence of NIDDM varies widely among racial groups) and environmental factors, such as diet, obesity and lack of physical exercise. The incidence of IDDM varies widely among countries and ethnic groups, for example in Finland it is ca. 10 times as high as in Japan and 60 times as high as in Mexico or China [3]. Both forms of the disease lead to serious complications such as neuropathy, nephropathy, retinopathy and premature atherosclerosis. A body of evidence is accumulating to suggest that tight blood glucose control is important in order to prevent or delay the onset of these complications and a large scale clinical study, the Diabetes Control and Complications Trial (DCCT), has been undertaken in order to provide a definitive answer to this question. The first results are now available [3a].

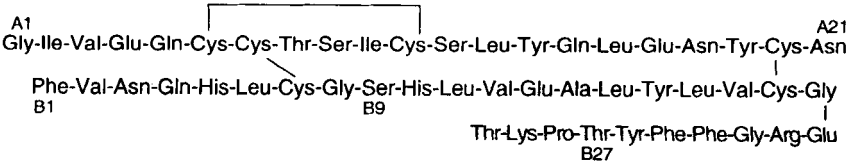
The goals of new therapeutic approaches for both IDDM and NIDDM have been and continue to be the provision of tight control both in the fasting and postprandial state and the correction of other metabolic abnormalities [4]. To be practically useful, this must be achieved without a significant risk of recurring hypoglycaemic episodes, a problem that plagues the existing form of therapies (sulphonylureas, insulin). New types of insulin therapy for IDDM patients are aimed at mimicking more closely the release of physiological insulin [5] and providing 24 h control.

This review will concentrate on new agents or new data on older agents reported in the past 5 years, and is confined to compounds with reported hypoglycaemic activity in man and/or animal models of IDDM or NIDDM. The patent literature has been recently reviewed [6] and will not be covered here. Established agents (sulphonylureas, biguanides) have been the subject of recent reviews and the reader is referred to those [4, 7–12].

INSULIN AND INSULIN ANALOGUES

INSULIN

Insulin is the primary therapy for all IDDM patients and is prescribed to NIDDM patients who do not respond to sulphonylureas or biguanides. Porcine insulin is slowly being replaced by human insulin (1), the majority of which is produced by recombinant DNA technology [13]. Some anecdotal reports of reduced hypoglycaemic awareness with the human protein [14–16] have been made public but were later criticized following subsequent studies [17–21] and a large scale clinical trial has been proposed in order to settle the issue [16, 22]. Although the safety of human insulin appears to be established, no significant clinical advantage has been



Human Insulin (1)

detected over animal insulin; therefore, it has been proposed that only newly diagnosed diabetics should be placed on the human version [23].

INSULIN ANALOGUES [24]

The search for insulin analogues has focused on compounds with different physical properties, aimed at mimicking the profile of endogenously released insulin [25, 26]. Thus a fast acting insulin analogue would be injected immediately before meals in order to mimic the insulin spikes in the postprandial state, while a long acting analogue could be taken once a day to provide 24 h basal insulin levels necessary for glucose regulation in the postabsorptive state.

Fast acting insulin analogues

Native human or porcine insulin molecules readily aggregate as hexamers. The rate of absorption after subcutaneous injection is dependent upon the dissociation of the insulin molecules into the monomeric state, resulting in a delay in the plasma appearance and a slow rise in plasma insulin levels. Peak concentrations of injected insulin are reached after 90–120 min instead of the 30–60 min peak observed after a meal in non-diabetic people [27]. Fast-acting analogues, when taken at mealtime, would mimic the rapid rise in endogenous plasma insulin seen in non-diabetics and allow the expected rapid glucose disposal [28–30]. Molecular modelling studies of the insulin molecule and its oligomers have allowed the identification of residues crucial for monomer–monomer interactions but with little influence on the binding of insulin to its receptor. Substituting negatively charged amino acids at this monomer–monomer interface for neutral amino acids (for example, B9Asp, B27Glu-insulin) introduces charge repulsion or weakens the hydrophobic interactions at the interface between monomers and decreases the tendency of the molecule to form oligomers. These monomeric insulin mutants are more soluble than the hexameric native peptide and are absorbed faster through subcutaneous tissue. Three analogues, X2 (B9Asp, B27Glu), X10 (B10Asp) and X14 (B28Asp) have been extensively studied in *in vitro* systems, in animals and in man. Their binding affinities (with respect to insulin) for the insulin receptor in HepG2 cells were 20%, 308% and 101% respectively [31]. In a euglycaemic clamp in the non-diabetic pig, equal amounts of X2 and X10 achieved the same overall effect on glucose utilization as human insulin [29]. In healthy volunteers X2 gave a similar fall in blood glucose as insulin and comparable plasma levels, consistent with a difference in clearance rates (the major pathway for insulin clearance being

Table 1.1. PROPERTIES OF FAST ACTING INSULIN ANALOGUES

	Human Insulin	*X2*	*X10*	*X14*
Binding affinity (%)	100[a]	20[a]	308[a]	101[a]
Clearance Rate per (ml/kg per min)	20[b]	7[b]	26[b]	NA
T_{50} (min)	180[b]	60[b]	90[b]	80[b]
GIR45(%max)	30[c]	83[c]	67[c]	64[d]

a: [31]; b: [35]; c: [33]; d: [30]; NA: not available.

internalization by its own receptor, less potent analogues clear more slowly) [32]. The absorption kinetics were studied in healthy men for X2 [33], X10 [33], and X14 [30]. As expected, X2 had a lower clearance rate than human insulin and X10. All three analogues were absorbed significantly faster, as evidenced by the T_{50} values (time for disappearance of 50% of the radioactive insulin at the injection site). The glucose infusion rates necessary to maintain euglycaemia after 45 min (GIR45) for all three analogues were significantly higher than for insulin, reflecting a faster onset of action.

Injection of X2 in diabetic subjects resulted in closer physiological levels (earlier and higher peak) than an equimolar amount of human insulin, due to the faster absorption and lower clearance rate [34]. Consequently, the compound has a larger effect than human insulin on reducing postprandial excursions when given immediately before meals. The reduced affinity (20% that of human insulin) leads to higher plasma levels, thus giving the analogue the same approximate *in vivo* potency as native insulin [35]. Similarly, injection of IDDM patients with X10 at meal-time gave a blood glucose control at least as good as human insulin administered 30 minutes earlier [36]. However, X10 was found to be carcinogenic in female rats [37], an effect believed to be due to enhanced affinity for the insulin-like growth factor I (IGF-1) receptor.

Another monomeric insulin was obtained by inverting the amino acids at positions B28 and B29. The analogue is absorbed more rapidly than human insulin and has a short duration of action and could be useful for controlling postprandial glucose levels [38]. B28Lys,B29Pro-insulin and B10Asp-des(B28-30)-insulin were also found to be rapidly absorbed in pigs while retaining insulin's efficacy [39]. Des(B27)-insulin, an analogue with 168% of insulin's biological potency *in vitro*, also caused a faster blood glucose drop in pigs than human insulin [40].

A different strategy to obtain monomeric or dimeric insulin is the glycosylation of the N termini, so that the attached carbohydrate residue

prevents aggregation. A1,B1-diglycosylated insulin and Bl-(1-deoxy-D-fructosyl)insulin lowered blood glucose in pigs faster than Actrapid™ insulin, the fastest insulin formulation available for therapy [41]. B1-(1-deoxy-D-fructosyl)insulin had *in vitro* potency similar to that of human insulin.

Long acting insulin analogues

These analogues are desirable in order to provide diabetic patients with once-daily injection to provide basal control of fasting plasma glucose. They can also be obtained by relatively simple amino acid mutations [42, 43]. Raising the isoelectric point toward 7 by introducing an amide at the end of the B chain and basic residues within the chains (for example, B27Lys, B30Thr-NH2) gave analogues with enhanced acid solubility but which crystallize once introduced into the physiological pH 7 medium, conferring prolonged action *via* slow dissolution. NovoSol Basal™, also named Insulin 174, (A21Gly, B27Arg, B30Thr-NH2) was compared in IDDM patients to the longest acting insulin available for therapy, Ultratard HM™ [44]. The T_{50} for NovoSol Basal™ (35.3 h) was significantly higher than that for Ultratard HM (25.5 h). In addition, Ultratard HM is a suspension which it is difficult to transfer into a syringe, leading to some variations in absorption. The soluble analogue NovoSol Basal™ provided lower inter- and intra patient variability. This may translate into a more reliable preparation and possibly a lower incidence of hypoglycaemic episodes. In a 2-week study with IDDM patients, NovoSol Basal™ provided a stable basal level of insulin after 24 h; however, the overnight glucose levels were somewhat higher than with Ultratard HM (10.4 vs. 7.3 mmol/l) [45]. This discrepancy can be explained by a difference in bioavailability of the two insulins, possibly because of degradation of the analogue after injection. In a study with 6 IDDM patients, the doses of NovoSol Basal needed to obtain the same control as that seen with Ultratard HM were two-fold higher [45]. A euglycaemic clamp in human volunteers showed NovoSol Basal to have 85% of the potency of human insulin [46]. Di-Arg (31-32) insulin (HOE 51 H), a natural biosynthetic precursor of insulin with similar *in vitro* potency [47], also gets released slowly after injection. In a euglycaemic clamp study this analogue had the same ability as insulin to affect glucose disposal and hepatic glucose output [48]. Similarly, A21Gly,B31Arg,B32Arg-insulin had a prolonged action profile in rabbits and dogs while maintaining the potency of insulin [49].

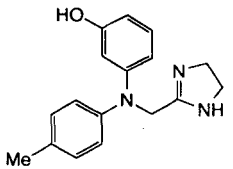
Proinsulin, the biosynthetic precursor of insulin, also has a long duration of action. Because of a difference in clearance rate, proinsulin is more active

at the liver in suppressing hepatic glucose output than in stimulating peripheral glucose disposal, a potential advantage for IDDM patients [50, 51]. However, apparent increases in myocardial infarction in one clinical trial caused development to be suspended [25].

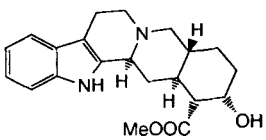
COMPOUNDS ACTING IN THE PANCREAS: INSULIN SECRETION

α_2-ANTAGONISTS AND IMIDAZOLINES

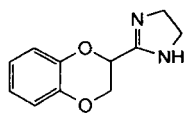
It was first proposed in 1976 [52] that sympathetic nervous overactivity plays a role in the defective insulin secretion of NIDDM and that therapeutic intervention at this level could be of benefit, by restoring the acute insulin response to glucose in type II diabetics [53]. Thus α-adrenoceptor blockade increases glucose-induced insulin release (GIR), while α-agonism blocks GIR. While phentolamine (2), a non-selective α-antagonist, potentiates GIR in isolated islets [54–56] and in animal models of NIDDM [54, 57, 58], prazosin has no effect, indicating that the pancreatic receptor is of the α_2-subtype. Similar effects have been observed in islets with yohimbine (3), idazoxan (4) [55, 56], efaroxan (6) [59] and with isaglidole (BDF 8933) (5) [60], and in the rat perfused pancreas with efaroxan (6) and RX821002A (7) [61].



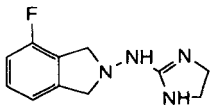
Phentolamine (2)



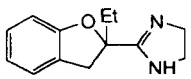
Yohimbine(3)



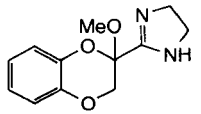
Idazoxan (4)



Isaglidole (5)



Efaroxan (6)

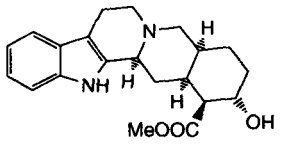


RX821002A (7)

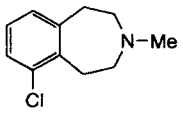
Animal Studies

In vivo GIR could be stimulated in normal rats with rauwolscine (8), SK&F 86466 (9) [62], idazoxan (4) [63, 64] and efaroxan (6) [65] and in diabetic

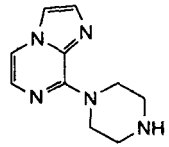
rodents with isaglidole (5) [66]. Conversely, α_2-adrenergic stimulation suppresses GIR as has been shown *in vitro* in islets [67] and *in vivo* in mice [68] with UK14304 or clonidine, and this effect can be reversed with an α_2-antagonist [65]. Thus, 8-(1-piperazinyl)imidazo[1,2-*a*]pyrazine (10), a selective α_2-antagonist [69], reversed the effects of clonidine in ob/ob mouse, a genetic model of NIDDM, by restoring glucose tolerance and lowering of blood glucose levels [70, 71], while efaroxan (6) could reverse the hyperglycaemic effect of UK14304 and adrenaline via increasing insulin levels [72].



Rauwolscine (8)



SKF-86466 (9)



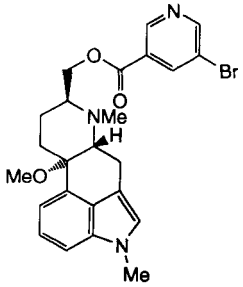
8-(1-Piperazinyl)imidazo-[1,2-a]pyrazine (10)

Compounds studied clinically

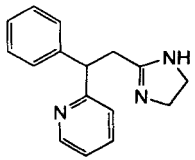
Phentolamine (2) was given to NIDDM patients and potentiated GIR without having any effect on the basal insulin secretion [73]. Similarly, NIDDM patients given nicergoline (11), a non-selective antagonist, underwent a restoration of the acute insulin response to glucose and an increase in the rate of disappearance of glucose [74].

Midaglizole (DG-5128) (12) is in development for the treatment of NIDDM. The compound lowered fasting plasma glucose levels and suppressed postprandial hyperglycaemia in laboratory animals [75]. In isolated islets [76] and in the rat perfused pancreas [77], it was shown that midaglizole improves both phases of insulin secretion in a glucose-dependent manner, while tolbutamide only affects the first phase, thereby providing a mechanistic difference from sulphonylureas. However the relevance of α_2-antagonism to the insulin releasing effect of midaglizole and other structurally-related compounds has been questioned (*vide infra*). In healthy subjects, the compound caused a substantial decrease in postprandial glucose levels even after a single dose [78]. Midaglizole restored postprandial insulin secretion and glucose disposal in NIDDM patients, also after a single dose [79]. In a 2- to 4-week study with 47 NIDDM patients, levels of fasting plasma glucose (FPG) and glycosylated haemoglobin (HbA_1) were decreased in subjects previously treated with diet

alone, while in patients previously on sulphonylureas FPG and HbA_1 remained at the same levels [80].



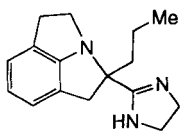
Nicergoline (11)



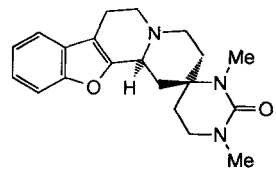
Midaglizole (12)

SL 84.0418 (13), a potent and selective α_2-antagonist [81], antagonizes the inhibitory effect on insulin release of UK14304 *in vitro* and in mouse or rat it stimulates GIR [82–84]. In the neonatal streptozotocin-treated diabetic rat, a NIDDM model, SL 84.0418 also increased the expression of the glucose transporter (Glut 4 isoform), indicating that the compound may improve peripheral insulin resistance in addition to its insulin-releasing property [85]. In healthy volunteers, a mild hypoglycaemic effect was observed while no cardiovascular parameter was affected [86, 87]. Following an oral glucose load in healthy subjects, SL 84.0418 inhibited blood glucose increase without increasing insulin levels, suggesting an extrapancreatic effect [88]. The (+)-enantiomer, deriglidole, is reported to be in phase II clinical trials for the treatment of NIDDM.

MK-912 (L-657,743) (14), an analogue of yohimbine with high *in vitro* potency and high α_2/α_1 selectivity [89, 90], lowers FPG and improves glucose tolerance in ob/ob mice and reversed the inhibition of GIR by clonidine [91]. In man, MK-912 attenuated the small elevation in blood glucose caused by clonidine, but had no significant effect on insulin concentration [92]. Healthy volunteers [93] and NIDDM patients [94] treated with MK-912 exhibited a small increase in basal insulin level and a modest decrease in FPG while substantial increases in catecholamines and



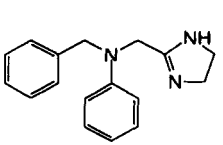
SL 84.0418 (13)



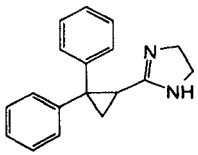
MK-912 (14)

blood pressure occurred. The compound also caused a slight improvement in GIR.

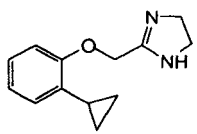
A series of observations has called into question the involvement of α_2-antagonism in the effect of some of the drugs above. Some antagonists such as yohimbine (3) can effectively block clonidine-induced inhibition of insulin secretion but are unable to increase secretion in the absence of added agonist [55, 94]. Ostenson observed in healthy and diabetic subjects that idazoxan (4) did not affect glucose-induced insulin release although complete blockade of α_2 activity at the β-cells had been achieved [95]. In rats, Schulz and Hasselblatt could induce GIR with phentolamine (2) but not rauwolscine (8), indicating an additional mechanism for phentolamine beyond α_2-adrenoceptor antagonism [96]. These same workers demonstrated a glucose-dependent increase in both phases of insulin release with antazoline (15), a compound closely related in structure to phentolamine but without any α_2-antagonistic activity. This effect could be blocked by clonidine, indicating that a group of imidazolines can affect insulin release independently of α_2-adrenoceptor blockade or agonism [97]. Other workers showed that cibenzoline (16), another imidazoline devoid of adrenergic activity [98], could stimulate GIR in rats [99] and in the rat perfused pancreas [61], while in the latter yohimbine (3) had no effect (this could explain the reported cases of cibenzoline-induced hypoglycaemia [99–101]). These results can be interpreted by invoking the presence on the β-cells of 'imidazoline-preferring' binding sites. In several tissue types, clonidine binds to a site from which it can be displaced by other imidazolines but not by yohimbine (3) nor by epinephrine (adrenaline) nor norepinephrine (noradrenaline) [102–104]. The relative lack of activity of idazoxan (4), an imidazoline, both in islets and in man [67], is readily explained by the heterogeneity of these sites [105–108]. Two distinct non-adrenergic sites have been uncovered for imidazolines; one, a clonidine-preferring site, tentatively called I_1, with the relative potency for displacing phentolamine: phentolamine > clonidine > idazoxan >> cirazoline (17); and a second, non-adrenergic idazoxan binding site (NAIBS or I_2) with the following ranking for the displacement of idazoxan: cirazoline > idazoxan > clonidine



Antazoline (15)



Cibenzoline (16)



Cirazoline (17)

[107]. The insulin-releasing effect of imidazolines would take place selectively via the clonidine-preferring site [109].

Another classification has been proposed based on the affinity of amiloride, a guanidine, which binds with high affinity at imidazoline sites in the human placenta [110]. Although the effector mechanisms of these proposed receptors are unknown, a putative endogenous ligand to the clonidine-preferring site, clonidine-displacing substance (CDS), has been characterized [111], while evidence suggests that phentolamine [112, 113], efaroxan [114, 115], midaglizole [116], and antazoline [112] increase insulin release by modulating ATP-sensitive potassium channels in β-cells.

GLUCAGON-LIKE PEPTIDES [117–119]

The gene for proglucagon encodes, in addition to glucagon (18), two structurally related peptides called Glucagon-like Peptide 1 (GLP-1) and Glucagon-like Peptide 2 (GLP-2) [120–122]. In the gut, GLP-1, a 37-amino acid peptide, is further processed into 2 smaller peptides, GLP-1(7-37) (20) (also called insulinotropin) and GLP-1 (7-36)-NH_2 (19), corresponding to the residues 7 to 37 and 7 to 36 of GLP-1, respectively, the latter peptide being amidated at the C terminus [123]. The biological properties of these two entities, as discovered thus far, are identical.

Glucagon (18)	**His**-Ser-Gln-**Gly**-**Thr**-**Phe**-**Thr**-**Ser**-**Asp**-Tyr-
GLP-1(7-36)-NH_2 (19)	^{7}**His**-Ala-Glu-**Gly**-**Thr**-**Phe**-**Thr**-**Ser**-**Asp**-Val-
GLP-1(7-37) (20)	^{7}**His**-Ala-Glu-**Gly**-**Thr**-**Phe**-**Thr**-**Ser**-**Asp**-Val-
Glucagon	-**Ser**-Lys-**Tyr**-Leu-Asp-Ser-Arg-Arg-**Ala**-Gln-
GLP-1(7-36)-NH_2	-**Ser**-Ser-**Tyr**-Leu-Glu-Gly-Gln-Ala-**Ala**-Lys-
GLP-1(7-37)	-**Ser**-Ser-**Tyr**-Leu-Glu-Gly-Gln-Ala-**Ala**-Lys-
Glucagon	-Asp-**Phe**-Val-Gln-**Trp**-**Leu**-Met-Asn-Thr
GLP-1(7-36)-NH_2	-Glu--**Phe**-Ile--Ala-**Trp**-**Leu**-Val-Lys--Gly-Arg-NH_2
GLP-1(7-37)	-Glu--**Phe**-Ile--Ala-**Trp**-**Leu**-Val-Lys--Gly-Arg-Gly

The similarity to glucagon and the location of secretion of these peptides suggested that they may be part of the enteroinsular axis, a hormonal system producing intestinal factors (incretins) in response to nutrients which influence the release of pancreatic hormones [124]. While GLP-1 and GLP-2 have no or little activity on hormone release from the pancreas, the truncated peptides GLP-1(7-37) and GLP-1(7-36)-NH_2 have potent glucose-dependent insulinotropic effects in the rat perfused pancreas [125–127] and in insulin-producing islet cell lines [128, 129]. In addition, GLP-1 (7-37) stimulates proinsulin synthesis, an important additional effect since it may then allow the constant replenishing of insulin stores and make exhaustion

of the β-cells unlikely [128, 130, 131]. The glucose-dependency is an important feature necessary in order to avoid hypoglycaemia *in vivo*; thus in an islet cell line, insulin release and biosynthesis take place at 25 mM glucose but not at 5 mM, while in the perfused pancreas insulin release was observed at 6.6 mM but not at 2.8 mM [128]. GLP-1 (7-36)-NH_2 also inhibits glucagon release in the perfused pancreas [132], although it is still unclear whether this is an effect of insulinotropin *per se* or is due to released insulin.

Infusion of GLP-1 (7-36)-NH_2 into healthy volunteers results in an enhancement of basal and glucose-induced insulin release, as well as a rise in insulin levels and a decline in glucagon levels [133, 134]. This is consistent with the insulin response to the rise in plasma glucose which is greater when glucose is administered orally than when injected intravenously, suggesting that GLP-1(7-36)-NH_2 is an incretin [135]. At basal plasma glucose concentrations GLP-1 (7-36)-NH_2 has no effect, indicating that the compound is unlikely to cause hypoglycaemic episodes [136]. In NIDDM patients, GLP-1 (7-36)-NH_2 significantly stimulates insulin release and reduces postprandial hyperglycaemia, while also lowering glucagon concentration [134, 137]. The decrease in glucagon is thought to be a direct action of the peptide. The peptide also reduces the amount of exogenous insulin needed to maintain normal glycaemia in a euglycaemic clamp study in both NIDDM and IDDM patients, suggesting an improvement in insulin sensitivity as well [134]. GLP-1(7-37) induced insulin release and lowered serum glucose without serious side effects in healthy volunteers [138, 139] and in NIDDM subjects [139, 140], including patients with secondary sulphonylurea failure [140]. The infusion of GLP-1(7-37) resulted in a 3- to 10-fold increase in peak plasma insulin levels both in non-diabetic and diabetic subjects and prevented the postprandial excursion in diabetic patients when given together with a meal. The insulin-releasing effect was glucose-dependent: pretreatment of NIDDM patients with insulin to lower fasting glucose levels resulted in attenuation of the response to GLP-1(7-37) [139, 140].

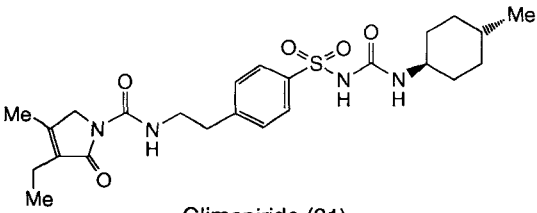
The mechanism of action of GLP-1(7-37) and GLP-1 (7-36)-NH_2 has been the subject of *in vitro* study. Both peptides increase *c*-AMP levels in insulin-producing cell lines in a dose-dependent manner [128, 141, 142] and functional receptors have been identified on insulin- and somatostatin-producing cell lines [142–144].

The insulinotropic activities of GLP-1(7-37) and GLP-1(7-36)-NH_2 are very structure-dependent, particularly in the N-terminal region. As mentioned above, GLP-1 is inactive. Deletion of the N terminus amino-acid (GLP-1 (8-37)-NH_2) or addition of the natural amino acid of GLP-1 at the

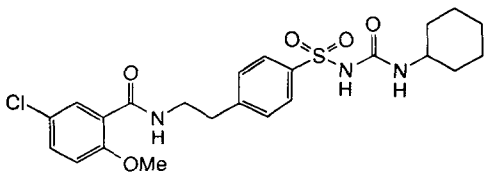
6 position (GLP-1 (6-37)-NH_2) produces peptides at least 100-fold weaker with respect to insulin-releasing and ability to increase *c*-AMP in the perfused pancreas. Deletion of amino acids at the C terminus has a less dramatic effect. GLP-1(7-35) and GLP-1(7-34) are 10-fold less active whereas GLP-1(7-33) has no effect [145, 146]. GLP-1(7-25) has no binding activity, whereas GLP-1(21-36)-NH_2 is a weak partial agonist [147]. It thus appears that both C and N termini are necessary for full biological activity.

NEW SULPHONYLUREAS AND SULPHONYLUREA ANALOGUES

Glimepiride (Hoe 490) (21), a new sulphonylurea, has a faster onset of action than glibenclamide (22) in three species (rat, dog, rabbit) and *in vitro* (rat perfused pancreas and perifused islets), a possible therapeutic advantage [148, 149]. Clinical studies with healthy volunteers showed that in the fasting state glimepiride lowers blood glucose levels without any significant increase in insulin release, suggesting an extrapancreatic effect [150], while in the fed state, the decrease in blood glucose levels is paralleled by a stimulation of insulin secretion [150, 151]. In NIDDM patients glimepiride exhibits significant hypoglycaemic activity and blunts the postprandial hyperglycaemic peak [152], and is as efficacious given once a day as glibenclamide given twice a day [153]. A once-daily sulphonylurea could result in better patient compliance.



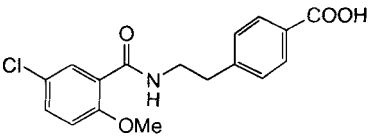
Glimepiride (21)



Glibenclamide (22)

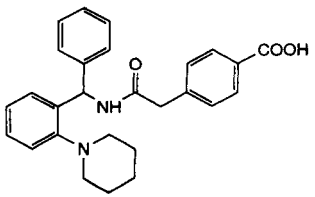
New compounds have emerged which are analogues of known active sulphonylureas where the sulphonylurea moiety has been replaced by a

carboxylic acid group. Meglitinide (HB 699) (23), a direct analogue of glibenclamide, stimulates insulin release *in vitro* in the perfused pancreas and lowers blood glucose in rats and dogs *via* an increase in insulin secretion [154]. In isolated islets meglitinide also increased insulin release, an effect which could be blocked by verapamil, a blocker of voltage-sensitive calcium channels, in a similar fashion to tolbutamide [155]. In dogs meglitinide is three times less potent than tolbutamide at inducing insulin release [156]. Meglitinide also inhibits active transport of glucose in the small intestine of rats [157]. The *in vitro* insulin-releasing effect has been shown to take place via the same mechanism as with sulphonylureas [158–163].

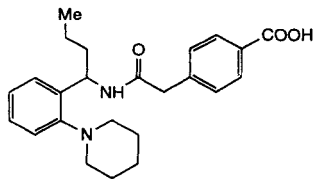


Meglitinide (23)

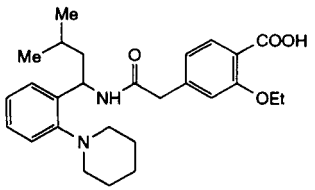
Another benzoic acid, AZ-DF 265 (24), stimulates insulin release in islet cells, also by the same mechanism as sulphonylureas, the activity residing in the laevorotatory enantiomer [164, 165]. A close-in analogue, AG-EE 86 (25), when administered to NIDDM patients, increases β-cell function with no effect on insulin sensitivity [166]. These two compounds are not being pursued further for pharmacokinetic reasons, but a third analogue, AG-EE 388 (26) is reported to be in phase II clinical trials [167]. Mechanistic studies on repaglinide (AG-EE 623 ZW) (27), the (*S*)-isomer of AG-EE 388, in isolated β-cells showed that the compound acts by closing ATP-dependent potassium channels like sulphonylureas [168]. In NIDDM patients, after 12 weeks, repaglinide was effective at lowering postprandial plasma glucose and is well tolerated [169]. Repaglinide was also effective at lowering post-prandial glucose after a single dose [170].



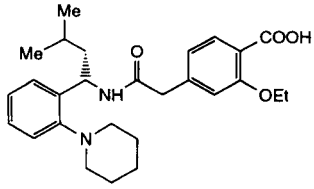
AZ-DF 265 (24)



AG-EE 86 (25)



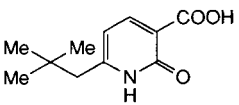
AG-EE 388 (26)



Repaglinide (27)

The insulin-releasing effect of sulphonylureas is thought to result from the closing of an ATP-dependent K^+ channel in the β-cell plasma membrane. The decrease in K^+ permeability leads to a depolarization of the membrane and activation of Ca^{2+} channels. Calcium then enters the cell and activates an effector system for the release of insulin [9, 158]. The receptor for the sulphonylureas is believed to be a part of the ATP-dependent K^+ channel or a protein which closely regulates that channel. A putative receptor has been characterized as a 140 kDa membrane protein by photoaffinity labelling with [^{3}H]-glibenclamide [171] or a radiolabelled derivative of glyburide [172]. The affinity at this binding site for glyburide was 0.36 nM, which correlates very closely with its ability to stimulate insulin (ED_{50} = 0.4 nM). The affinities of various sulphonylureas and meglitinide analogues also correlate with their insulin-releasing properties and their K^+ channel blocking potencies [162]. These results are consistent with the characterized binding site being the receptor for sulphonylureas and with meglitinide having a common mechanism of action with the sulphonylureas.

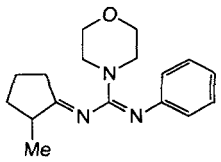
U-56324 (28), a nicotinic acid derivative, has hypoglycaemic activity in the 18-h fasted normal rat [173] and stimulates *in vitro* insulin secretion [174]. Study of the *in vitro* activity in membrane patches from cultured mouse pancreatic β-cells revealed that it acts directly on ATP-sensitive potassium channels and probably has the same mechanism of action as sulphonylureas [174].



U-56324 (28)

OTHER INSULIN SECRETAGOGUES

Linogliride (29) is a substituted guanidine structurally unrelated to the sulphonylureas but with a similar mechanism of action. Linogliride potentiates insulin release in isolated islets [175] and in the perfused pancreas [176, 177]. In islets, linogliride is reported to stimulate insulin secretion in the presence of glucose but not in its absence [175], and unlike tolbutamide it produces a larger insulin second phase release without a significant first phase peak [178]. These results argue for a mechanistic distinction from the sulphonylureas. However it was found that in the rat perfused pancreas insulin secretion could be triggered in the absence of glucose [177]. Moreover the process is calcium-dependent [175, 176] and proceeds via inhibition of ATP-sensitive potassium channels [179], indications that linogliride and the sulphonylureas act via closely related mechanisms. Possible extrapancreatic effects of the agent have also been suggested [176, 180].



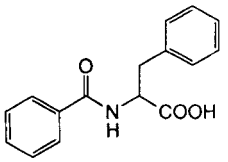
Linogliride (29)

Linogliride produces hypoglycaemic effects in various non-diabetic and diabetic animals [181]. In normal rats and dogs, it improves glucose tolerance and in fasted rats or mice, linogliride lowers blood glucose. Linogliride showed modest effects in the db/db mice (an insulin resistant genetic model of NIDDM in which sulphonylureas are inactive) but significantly lowered fasting blood glucose in neonatal streptozotocin-treated rats [182].

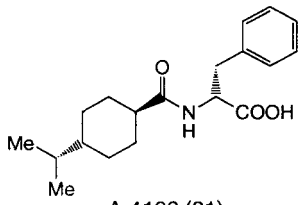
Clinical studies with NIDDM patients have shown linogliride to be effective at lowering fasting and postprandial plasma glucose levels in NIDDM patients [183–185]. The lowering of plasma glucose is associated with an increase in insulin levels, consistent with an insulin secretagogue mechanism. Long-term clinical studies have demonstrated the efficacy and safety of linogliride as an oral hypoglycaemic agent [176]. However the compound was withdrawn from development due to preclinical safety issues [176].

Researchers at Ajinomoto set out to discover new hypoglycaemic compounds by screening a large number of compounds at a single dose in

18-h fasted normal mice [186]. Their lead compound, *N*-benzoyl-DL-phenylalanine (30) was weakly active at 500 mg/kg. Modification of the benzoyl group into a 4-substituted cyclohexyl amide and separation of the stereoisomers led to the discovery of a more potent compound which had significant hypoglycaemic activity at 10 mg/kg, *N*-[(*trans*-4-isopropylcyclohexyl)carbonyl]-D-phenylalanine (A-4166) (31) [187].



N-Benzoyl-DL-phenylalanine (30)



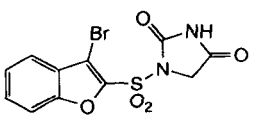
A-4166 (31)

A-4166 was able to effect a 20% blood glucose drop at 1.6 mg/kg after a single dose. In fasted dogs, the decrease in blood glucose started after 45 min., was maximal after 60 min. and levelled off after 5 h. This effect was accompanied by a rise in plasma insulin level [188, 189]. This fast and short-term action was also observed in rats and may have important implications for human therapy [189]: a compound having a rapid and short insulin-releasing effect after a single dose could be effective in preventing postprandial hyperglycemia without causing hypoglycaemia. Indeed when compared with tolbutamide, glibenclamide and glipizide in fasting rats after a glucose challenge, A-4166 was equally efficacious in preventing the glycaemic excursion but unlike the sulphonylureas did not cause prolonged hypoglycaemia, even at the highest dose (100 mg/kg). Repeated administration over 2 weeks produced the same effect on a given day as single administration. The compound was also active in two diabetic models, the KK mouse and the streptozotocin-treated rat, also after a single dose (tolbutamide is inactive in the KK mouse). After 30 days toxicology in rat, no changes were observed in the liver, kidney and pancreas of the animals [190].

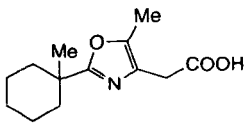
In islets, A-4166 causes a steep rise in insulin release followed by a slow sustained rise to twice the basal level. The insulinotropic effect is not glucose-dependent and takes place in the absence of glucose [191, 192]. The *in vivo* pharmacodynamic profile (rapid and short-term action) appears to result from a rapid plasma appearance and disappearance of the compound rather than an intrinsic feature of the mechanism [191]. A-4166 may be useful as therapy for NIDDM patients with secondary failure to sulphonylureas [190].

M16209 (32), a compound first discovered as an aldose reductase inhibitor [193, 194], was found to possess hypoglycaemic activity at high dose in the streptozotocin diabetic rat and to improve glucose tolerance in the normal rat and the neonatal streptozotocin diabetic rat, a model of NIDDM. The compound elevates insulin levels in hyperglycaemic rats but has no effect on glucose or insulin levels in normal animals, suggesting that its mechanism involves potentiation of GIR [195]. Indeed, in the rat perfused pancreas, M16209 can induce insulin secretion at high glucose concentration (11.1 mM) but not at low concentration (5.6 mM), unlike gliclazide, a sulphonylurea used for comparison in the study [196]. This shows that M16209 is a glucose dose-dependent insulin secretagogue, a property that may reduce the occurrence of hypoglycaemic episodes.

A series of 4-oxazoleacetic acid derivatives was identified as a group of potential hypoglycaemic agents in mice via the insulin tolerance test [197]. The most potent compound, AD-4610 (33), was found to potentiate biphasic glucose-induced insulin secretion in the perfused pancreas and in normal rats in a glucose-dependent fashion. AD-4610 also lowers plasma glucose in yellow KK mice, a model of NIDDM in which tolbutamide is inactive [198]. Development of AD-4610 and/or analogues is reported to have been discontinued [167].



M16209 (32)



AD-4610 (33)

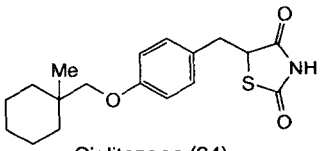
COMPOUNDS ACTING IN MUSCLE

This section describes agents which either modulate or mimic insulin's actions in muscle, its main target tissue for glucose disposal [199–201]. Insulin resistance, that is, the defect in the insulin stimulation of glucose uptake by muscle and fat and in the inhibition of hepatic glucose output (HGO), has become recognized as a major contributor to the NIDDM pathology [200–202], and may in some patients precede overt diabetes by many years [203]. Agents which simulate or improve the response to insulin and lower plasma glucose without stimulating insulin release [204] would directly lower insulin resistance and could circumvent the problems associated with insulin-releasing agents, that is, primary and secondary

failure, and hypoglycaemic episodes. Most of the compounds stimulating glucose uptake are reported to have liver effects as well.

CIGLITAZONE-TYPE COMPOUNDS

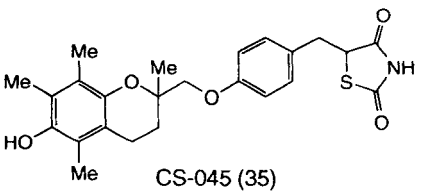
In 1982, Takeda, Inc. disclosed a series of 5-benzylthiazolidinediones as a new class of hypoglycaemics [205]. These compounds, and in particular the prototype ciglitazone (34), lower blood glucose in NIDDM animal models without affecting insulin release, that is, reduce the insulin resistance inherent in these obese diabetic animals [206, 207].



Ciglitazone (34)

Animal studies

Since the discovery of ciglitazone, scientists at Takeda as well as other companies have successfully expanded the structure–activity relationships of the molecule and identified more potent analogues, using genetically diabetic mice as animal models of NIDDM. Scientists at Sankyo attached the benzylthiazolidinedione portion of the molecule to a vitamin E-like tail in order to combine the hypoglycaemic activity with a lipid peroxide lowering effect. In this way, they obtained CS-045 (35) (ED_{25} = 6 mg/kg) and other derivatives, using the KK-mouse as a model [208].

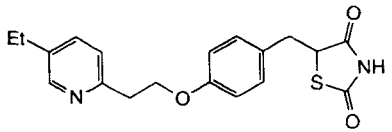


CS-045 (35)

In the ob/ob mouse, the KK mouse and the Zucker rat, CS-045 reduces the levels of blood glucose, insulin, triglycerides, lactate, and free fatty acids, while in the normal rat and the streptozotocin-diabetic rat, no effect on blood glucose is observed. The effect on blood glucose in KK mice can be observed after a single 50 mg/kg dose within 2.5 hours and lasts as long as

18 hours [209]. Part of this effect is due to suppression of hepatic gluconeogenesis, as evidenced by uptake of $NaH^{14}CO_3$ into blood glucose [210]. CS-045 also improves hyperglycaemia and glucose tolerance in db/db mice at a late stage of the diabetic syndrome, with a slowing of islet degeneration [211].

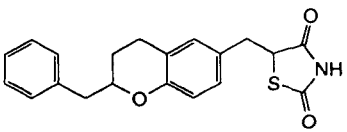
Takeda researchers improved on the potency of ciglitazone (ED_{25} = 40 mg/kg) by replacing the methylcyclohexylmethyl tail by a 2-pyridinylethyl moiety, and showed that the nitrogen at the 2-position of the pyridine ring is crucial in the potency gain [212, 213]. Among the most potent compounds, pioglitazone (36) (ED_{90} = 6 mg/kg), was selected for development based on further toxicological evaluation. Pioglitazone has similar effects to CS-045 on KK mice, Zucker rats [214] and streptozotocin-diabetic rats [215] and improves insulin sensitivity in obese dogs [214], Wistar fatty rats [216] and insulin-resistant rats [217] as well.



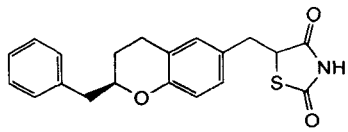
Pioglitazone (36)

In Wistar fatty rats, pioglitazone administered over 6 days improves both insulin's suppression of hepatic glucose output and its ability to stimulate glucose utilization, indicating that the compound acts by reducing insulin resistance both at the liver and in peripheral tissues [218]. Pioglitazone, in conjunction with insulin but not by itself, inhibits cholesterol absorption and lowers plasma cholesterol in cholesterol-fed rats, producing a two-fold decrease of the ratio of total cholesterol to HDL cholesterol [219]. In Wistar fatty rats, pioglitazone lowers cholesterol and non-esterified fatty acids levels [220].

The central portion of the ciglitazone molecule can also be modified: changing the phenyl ether to a dihydrobenzofuran or dihydrobenzopyran and adding a benzyl tail at the 2-position produced compounds such as CP-68,722 (37), later resolved to the (*R*)-isomer, englitazone (38), which had



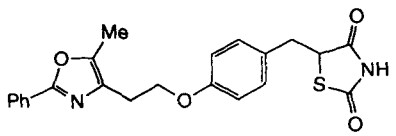
CP-68,722 (37)



Englitazone (38)

significant blood lowering effects in ob/ob mice at 5–10 mg/kg [221]. CP-68,722 lowers levels of glucose and insulin in ob/ob mice without producing hypoglycaemia, the drop in glucose preceding that in insulin, an indication that the effect on insulin is secondary [222]. A euglycaemic clamp study in the Zucker rat showed that the enhancement in insulin sensitivity is due to effects on both glucose disposal and hepatic glucose output and that insulin clearance is increased as well [223]. In normal rats, englitazone has no effect on plasma glucose but lowers basal plasma insulin levels, indicating that the compound improved insulin sensitivity even in non-diabetic animals, thus reducing the insulin requirement [224]. Englitazone also lowers plasma cholesterol in cholesterol-fed mice [225].

Extremely potent compounds can be obtained by further elaborating the pyridine ring of pioglitazone into an oxazole ring. Thus, a series of 4-oxazolylethyl derivatives was found to have very strong hypoglycaemic activity in the KKA^y mouse, the most active compound (39) having an ED_{25} of 0.09 mg/kg [226]. Similarly, the ketone darglitazone (CP-86,325) (40) normalizes blood glucose in ob/ob mice at 0.5 mg/kg, thereby demonstrat-



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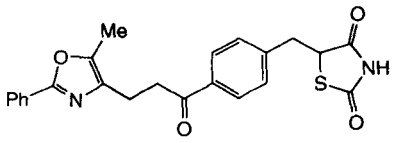
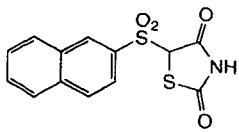


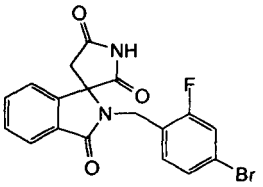
Darglitazone (40)

ing that the ether function of the previous compounds is not essential for the activity [227]. 5-Arylsulphonyl thiazolidinediones such as (41) have also been prepared and showed to lower blood glucose in db/db mice and the Zucker rat, (41) being equipotent to ciglitazone in these models [228]. A series of spirosuccinimide aldose reductase inhibitors such as (42) and (43) which also have hypoglycaemic activity comparable with that of ciglitazone have been recently reported [229]. The series was not pursued further because of the low potency and the inability of the compounds to reduce insulin levels as well, a property which all active thiazolidinediones appear



AY-31,637 (41)

to possess. That these compounds lower blood glucose by a ciglitazone-like mechanism is unclear at present.



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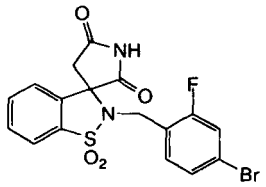
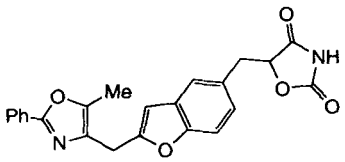


(43)

The thiazolidinedione group can be replaced by other acidic heterocycles. Oxazolidinediones such as (44) can achieve high potency [230]. In the ob/ob mouse (44) normalized blood glucose at 0.5 mg/kg. The thiazolidinedione can also be replaced by an oxathiadiazole oxide. The 2-naphthyl compound (45), analogous to the sulphonyl thiazolidinedione (41), was the most active compound of the series, ca. 5 times as potent as ciglitazone [231].



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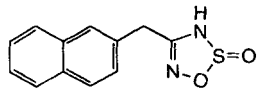
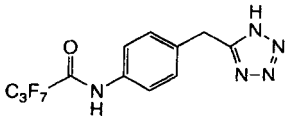
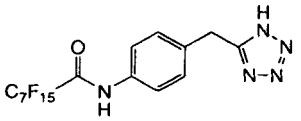


(45)

Researchers at Wyeth-Ayerst have successfully replaced the thiazolidinedione ring of ciglitazone by a tetrazole and the lipophilic portion by a perfluoroalkyl chain [232]. These compounds have a similar biological profile to ciglitazone [233]; however, fundamental differences are apparent in the structure–activity relationships. Whereas the acidic functionality of the thiazolidinedione ring and its close analogues appears to be essential for activity, the tetrazole can be methylated or removed altogether with retention of most of the hypoglycaemic activity. Indeed in this series it appears that the perfluoro chain is the critical structural component controlling the potency [232]. Two compounds were selected for additional studies, Wy-49,146 (46) and Wy-49,322 (47). The glucose-lowering by these two agents in ob/ob and db/db mice is observed 2 days after dosing and is accompanied by a fall in insulin levels. In normal rats, no hypoglycaemic effect is observed but, with the more potent Wy-49,322 only, plasma insulin, cholesterol, and triglyceride levels are decreased. However, unfavourable



Wy-49,146 (46)



Wy-49,322 (47)

side-effect profiles precluded further development of these compounds [233].

Mechanism of action

While it is clear that the mechanism of action of ciglitazone (34) and its derivatives does not involve the release of insulin, the molecular target(s) is (are) still unknown. Various studies with CS-045 (35), pioglitazone (36) and englitazone (38) have examined the peripheral (muscle, fat) and liver effects of these agents. Peripheral insulin resistance manifests itself by defects in the glucose transport system. Fat cells taken from NIDDM patients display a decrease in their maximal rate of insulin-stimulated glucose uptake [234]. In the 3T3-L1 adipocyte cell line, CP-68,722 (37) stimulates both basal and insulin-stimulated glucose uptake, thus displaying an insulinomimetic and insulin-enhancing action, without any change in insulin-binding. The increase in uptake could be blocked by cycloheximide, a protein synthesis inhibitor, indicating that protein synthesis is involved in this action [235]. Indeed, CP-68,722 increases expression of the glucose transporter protein in this system, thus explaining the dual insulinomimetic and insulin-enhancing effects. CP-68,722 and insulin display additive effects on glucose transporter expression and synergistic effects on glucose transport [236]. Similarly, pioglitazone enhances basal and insulin-stimulated glucose transport in the BC3H-1 muscle cell line [237]. In soleus muscle, it increases insulin-stimulated glycogen synthesis and glycolysis and in adipocytes glucose oxidation and lipogenesis [216]. Thiazolidinediones have also been shown to improve these defects *in vivo*. Adipocytes isolated from KK mice or Zucker rats treated with CS-045 show an improvement in insulin responsiveness, as evidenced by glucose uptake and insulin binding [209]. Using soleus muscle isolated from pioglitazone-treated insulin-resistant rats [217] or streptozotocin-diabetic rats [238], glucose transport function was restored to normal. In the latter study, levels of glucose transporter mRNA were found to be restored to that of control non-diabetic mice. The insulin-sensitive Glut4 glucose transporter isoform was increased whereas englitazone in 3T3-L1 cells increases expression of Glut1 but not Glut4 [239]. Pioglitazone

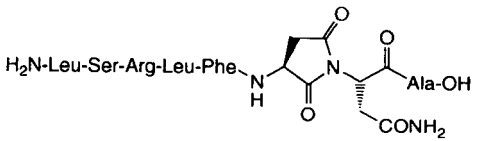
treatment also activates the tyrosine kinase activity of the insulin receptor in muscle cells with respect to autophosphorylation as well as exogenous substrate phosphorylation [240]. Non-diabetic animals also show changes in insulin action after thiazolidinedione treatment: adipocytes from normal rats treated with CP-68,722 displayed increases in basal and insulin-stimulated glucose uptake [224]. Liver effects can also be observed *in vitro* and *in vivo*. CS-045 suppresses glucose production from lactate in the perfused liver [210]. Hepatocytes isolated from pioglitazone-treated KK mice are more responsive to insulin than those from untreated animals with respect to the activities of three insulin-regulated enzymes: pioglitazone restores the activities of glucose-6-phosphate dehydrogenase, glucose-6-phosphatase and phosphoenolpyruvate carboxykinase to the levels found in non-diabetic mice [241, 242].

Clinical studies

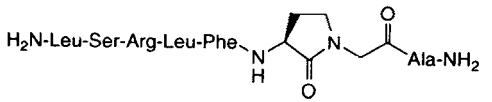
In an open study with 146 NIDDM patients, CS-045 (35) at 200 mg/day for 12 weeks produced a reduction in fasting plasma glucose (FPG) in 25% of the patients, whereas at 400 mg/day, 46% of the patients displayed an equivalent improvement in FPG [243]. The rate of response was higher in obese patients than in lean patients. In a separate study, 216 NIDDM patients (FPG > 150) were dosed with 400 mg/day CS-045 for 8 weeks at which time some of the non-responders (FPG > 140) received 800 mg/day for another 8 weeks [244]. The patients who showed a response at 8 weeks showed no additional FPG drop after the additional 8 weeks (176 mg/dl to 121 mg/dl to 133 mg/dl); the non-responders having received 800 mg/day for the last 8 weeks showed only a slight improvement after the dose increase (202 mg/dl to 187 mg/dl to 178 mg/dl); the non-responders kept on 400 mg/day for the whole 16 weeks showed no improvement (194 mg/dl to 167 mg/dl to 173 mg/dl). These results indicate that an increased dose failed to enhance the effect of the compound. Finally, in an open 11-patient study at 400 mg/day for 6–12 weeks, the group divided into 8 responders (FPG from 230 mg/dl to 149 mg/dl) and 3 non-responders (FPG from 220 mg/dl to 248 mg/dl). The blood-glucose lowering started after 2–3 weeks and reached maximum after 6–11 weeks. Insulin levels were also lowered (30 μU/ml to 16 μU/ml for the whole group), indicating an improvement in the patients' insulin resistance. Postprandial glucose levels were decreased as well. Hepatic glucose output decreased by 17% in the total group and 28% in the responders group. The glucose disposal rate was improved in every patient, indicating that the compound has effects both at the liver and in the peripheral tissues in man [245].

HUMAN GROWTH HORMONE FRAGMENTS

Human growth hormone (hGH), a 191-amino acid protein, is known to possess both insulin-like and anti-insulin actions *in vitro* and in animals [246]. Multiple fragments of hGH, particularly in the amino-terminal region, have been reported to possess insulin-like or insulin potentiating activity, raising the possibility that hGH functions as a prohormone which releases biologically active small peptides to regulate insulin's action. Ng *et al.* were the first to demonstrate the hypoglycaemic activity of peptides prepared by limited hydrolysis of hGH [247]. Among the fragments obtained, hGH-(8-13) (Arg-Leu-Phe-Asp-Asn-Ala) was the smallest active sequence and hGH-(6-13) (Leu-Ser-Arg-Leu-Phe-Asp-Asn-Ala) (48) was selected for further studies. It was found subsequently that the aspartic acid residue of hGH-(6-13) is in its cyclic succinimide form, an essential feature required for activity. hGH-(6-13) increases insulin binding to hepatic plasma membranes and adipocytes by increasing the number of active receptors and/or their affinity for insulin and facilitate glucose transport in isolated cells. *In vivo*, hGH-(6-13) increases glucose clearance as evidenced in the oral i.v. glucose tolerance test and enhances tissue sensitivity to insulin action [247]. A more stable analogue was recently discovered where the succinimide ring has been replaced by a γ-lactam ring. The new compound, γ-lactam[11] hGH-(6-13) (49), has similar biological properties to hGH-(6-13) with a longer duration of action, due to improved stability and possibly to enhanced bioavailability [248, 249].



hGH(6-13) (48)



γ-Lactam[11]-hGH(6-13) (49)

hGH-(1-43) has been isolated as a component of pituitary glands. The insulin sensitivity of adipocytes of A^{vy}/A obese diabetic mice treated with

this fragment is markedly improved [250]. *In vivo*, hGH-(1-43) increases insulin-induced glucose clearance in yellow mice [251, 252] and ob/ob mice [253] without any direct effect on insulin or blood glucose levels.

An endogenous human growth hormone fragment, hGH_{20K}, has been discovered that lacks the insulin-like activity of the 191 amino-acid protein, hGH_{22K}, while retaining its growth-promoting activity [254]. The missing fragment, hGH-(32-46) is reported to increase insulin and glucagon levels under euglycaemic conditions and to double the insulin response in the hyperglycaemic state in conscious dogs [255]. *In vitro*, the peptide decreases hepatic glucose output in the perfused liver and increases glucose uptake in muscle cells, suggesting an insulin-potentiating action as well [256]. A shorter fragment, hGH-(32-38), displays an insulin-enhancing action *in vivo*: hGH-(32-38) is inactive in dogs under euglycaemic conditions and increases glucose clearance during hyperglycaemia without any effect on glucagon or insulin release [257].

VANADIUM COMPOUNDS

Vanadate (VO_3^- or $H_2VO_4^-$) was first recognized in 1979 as having insulin mimetic properties [258]. Since then, vanadate and vanadyl (V^{4+}) have been shown to mimic most but not all biological actions of insulin *in vitro* and to lower blood glucose in streptozotocin-treated rats [259, 260]. Vanadate is a potent inhibitor of phosphotyrosine phosphatases, an interesting activity since the insulin receptor is a tyrosine kinase, and some of the actions of insulin have been proposed to take place via autophosphorylation of the insulin receptor and phosphorylation of cellular substrates on tyrosine residues [261]. Some recent developments on the mechanism and the *in vivo* activity of vanadate and its derivatives are presented here.

In vitro *studies*

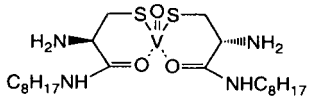
Evidence has been provided showing that some of the insulin-mimicking actions of vanadate could be elicited without any effect on protein phosphorylation [262, 263]. Eriksson *et al.* showed that vanadate in rat adipocytes increases insulin binding by increasing the receptor number, without changing the affinity of insulin, and stimulates glucose transport into the cell. Whether this effect is linked to tyrosine phosphorylation is unclear [264].

In vivo *studies*

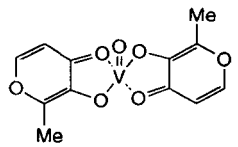
Vanadate has been recently evaluated in various NIDDM models, and for extended periods of time. In the ob/ob mouse, administration of vanadate for 3 weeks lowered blood glucose levels to control values without any signs of hepatotoxicity or any effect on body weight [265]. In another study, after 7-week treatment of ob/ob mouse, an i.v. glucose test showed that the glucose disappearance rate had increased two-fold while the hepatic glycogen content had also increased two-fold [266]. Improvement in glucose tolerance was also observed in fa/fa rats, an insulin resistant model of NIDDM, after 3 months treatment [267]. The antidiabetic effect of vanadium salts persists after the end of the treatment: in streptozotocin-treated rats, 3-week treatment with vanadyl sulphate was followed by a 13-week withdrawal period, at the end of which the animals were still normoglycaemic and the pancreatic islets had been protected against the usual degenerative effects of streptozotocin administration [268]. Hyperinsulinemic euglycaemic clamp studies have shown that an increase in glucose utilization is the main factor responsible for the improvement in glycaemia [269–272], although some studies have detected an effect on glucose production as well [271, 272].

Vanadium complexes

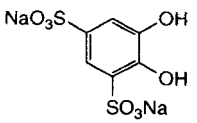
Several groups have attempted to increase the therapeutic index of inorganic vanadium salts by preparing complexes with organic chelating agents. Naglivan (50), a vanadium (IV) complex, is less toxic than vanadium sulphate and is effective in lowering the hyperglycaemia of streptozotocin-



Naglivan (50)



Bis(maltolato)oxovanadium (51)

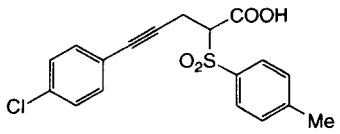


Tiron^R (52)

diabetic rats at 50 mg/kg and db/db mice at 5 to 20 mg/kg [273]. Similarly, bis(maltolato)oxovanadium (51), also a vanadium (IV) complex, lowers the blood glucose and cholesterol levels in streptozotocin-diabetic rats to within the normal range at a lower dose than vanadyl sulphate without any detectable toxicity or dehydration [274]. Another approach, namely, administering Tiron^{R} (52), a metal chelator, i.p. in addition to oral vanadate, lowers renal toxicity while maintaining vanadate's insulin-mimicking properties in streptozotocin-diabetic rats [275].

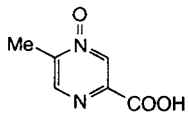
MISCELLANEOUS COMPOUNDS

BM 13.0907 (53) lowers blood glucose in ob/ob mice by a mechanism closely resembling that of insulin. In rat adipocytes, BM 13.0907 increases glucose uptake and stimulates the translocation of glucose transporters from intracellular compartments to the plasma membrane [276]. In ob/ob mice, BM 13.0907 normalizes blood glucose at 50 mg/kg *p.o.* Adipocytes isolated from treated animals display an increase in glucose uptake and an increased ability to translocate glucose transporters [277].



BM 13.0907 (53)

Acipimox (54), a potent and long-acting antilipolytic agent, increases insulin sensitivity in NIDDM patients, an effect consistent with the hypothesis that non-esterified fatty acids contribute to the insulin resistance of these patients. In hyperinsulinaemic euglycaemic clamp studies, prolonged exposure to acipimox resulted in an increase in the rate of glucose utilization; however, in the basal state glucose utilization was unchanged [278, 279]. A lowering of hepatic glucose output was also reported [279].



Acipimox (54)

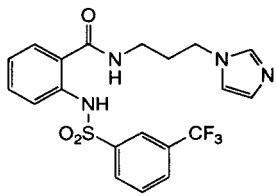
COMPOUNDS ACTING IN THE LIVER

Excessive hepatic glucose output is an important contributing factor to insulin resistance [200, 201, 280] and direct or indirect inhibition of glucose production is expected to have a favourable effect on hyperglycaemia, particularly in the fasting state.

GLUCAGON SECRETION INHIBITORS

Glucagon is the primary counter-regulatory hormone responsible for stimulating glucose output. Hyperglucagonaemia is a common characteristic of IDDM and NIDDM patients [281, 282] and inhibition of glucagon secretion, an important action of insulin itself [283], is an attractive approach for lowering hyperglycaemia.

M&B 39890A (55) has been found to decrease basal and arginine-stimulated glucagon secretion in rat isolated islets [284] and in the perfused pancreas [285]. However, it also inhibits insulin secretion in these two systems, a potentially undesirable effect [285, 286]. In fed VY/WfL-A^{vy}/a mice, an obese NIDDM model, treatment with M&B 39890A for 3 days lowered blood glucose and insulin levels, while no effect was observed in fasted diabetic mice, normal mice or streptozotocin-diabetic rats. Chronic treatment of fed mice for 42 days reversed the hyperglycaemia without tachyphylaxis and improved insulin sensitivity. In addition, the compound prevented the development of hyperglycaemia in prediabetic mice [286].

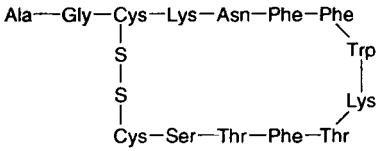


M&B 39890A (55)

SOMATOSTATIN ANALOGUES

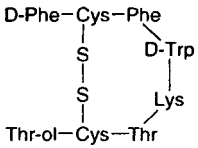
Somatostatin (56), a 14-amino acid peptide, inhibits secretion of glucagon and growth hormone and decreases carbohydrate absorption [287, 288]. It also inhibits insulin secretion, a property which is not relevant to IDDM

therapy but which is undesirable for the treatment of NIDDM. Analogues which would inhibit glucagon and/or growth hormone secretion without affecting insulin release are expected to have a positive effect on glucose control and the long-term consequences of diabetes, since growth hormone has been implicated in some of the chronic complications of diabetes [289–293].



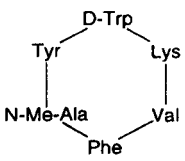
Somatostatin (56)

Octreotide (SMS 201-995, Sandostatin^{R}) (57), an octapeptide analogue [294], lowers growth hormone and glucagon levels, reduces postprandial hyperglycaemia and lowers the insulin requirement in IDDM patients [295–298]. In insulin-treated NIDDM patients, octreotide lowers postprandial glycaemia [299]. Side-effects have been reported in a few of the studies: dose-limiting gastrointestinal effects [300], hyperalaninaemia [297], and a few instances of hypoglycaemic episodes [298].

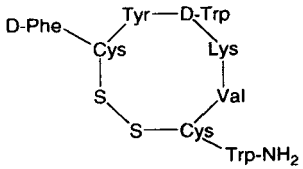


Octreotide (57)

MK-678 (L363,586) (58), a cyclic hexapeptide, lowers fasting and postprandial hyperglycaemia in IDDM patients, after i.v. or intranasal delivery [301], and, taken intranasally at bedtime, can abolish the morning



MK-678 (58)



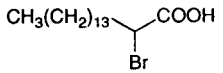
RC-160 (59)

hyperglycemia (dawn phenomenon), presumably via lowering of growth hormone levels [302].

A newer agent, RC-160 (59), was tested in streptozotocin-diabetic rats. Injection of RC-160 subcutaneously inhibits glucagon release and lowers plasma glucose levels [303].

FATTY ACID OXIDATION INHIBITORS

NIDDM is characterized by excessive fatty acid oxidation. The availability of excess substrates for glucose production results in enhanced gluconeogenesis and impaired glucose oxidation [304]. Inhibition of fatty acid oxidation should reduce gluconeogenesis and ketogenesis (the formation of ketone bodies), activate glycolysis and lower fasting glycaemia. The discovery of 2-bromopalmitate as an inhibitor of fatty acid oxidation [305] led to the study of these types of agents as possible hypoglycaemics.



2-Bromopalmitic acid (60)

2-Bromopalmitate (60) and subsequent fatty oxidation inhibitors block the carnitine-dependent transport of fatty acids across the mitochondrial membrane (*Figure 1.1*). Fatty acids are activated by conversion to their CoA derivatives, transported through the outer membrane, transformed into their carnitine esters by the outer-membrane carnitine palmitoyl-CoA transferase (CPT 1), transferred through the inner membrane by a carrier called carnitine acylcarnitine translocase, then regenerated as the free acids by the inner-membrane carnitine palmitoyl-CoA transferase (CPT II) before undergoing the β-oxidation sequence. Inhibitors of both CPT I and carnitine acylcarnitine translocase have been developed as hypoglycaemic agents.

CPT I Inhibitors [306, 307]

Using 2-bromopalmitate (60) as a lead, scientists at Byk Gulden discovered a series of ω-aryl-alkylglycidic acids [308], while McNeil Pharmaceuticals researchers found a series of alkylglycidic acids as CPT I inhibitors and hypoglycaemic agents [309]. Both groups found the glycidate motif to be critical for the biological activity. Methyl palmoxirate (61), clomoxir (62) and etomoxir (63) all require *in vivo* activation *via* ester hydrolysis and

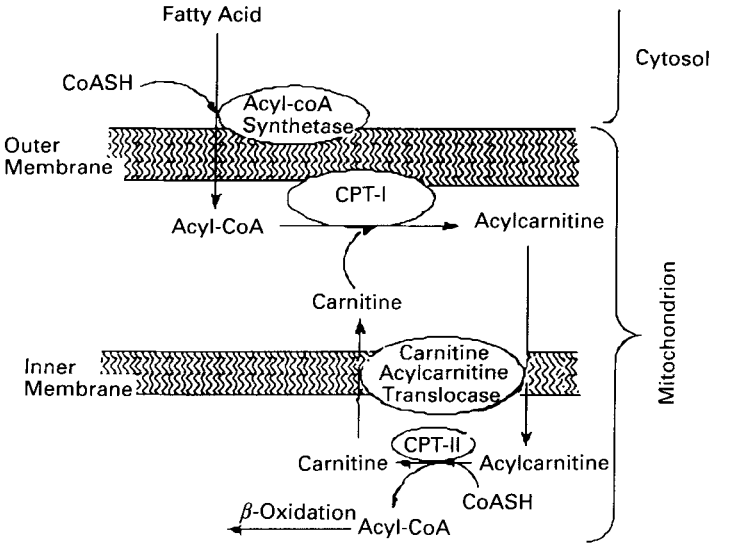
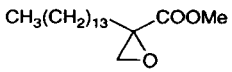
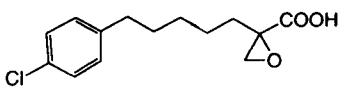


Figure 1.1. Fatty acid transport across the mitochondrial membrane

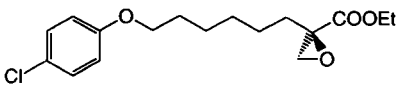
formation of the acyl-CoA derivatives for the inhibition to take place. In liver tissue isolated from streptozotocin-diabetic rats treated with etomoxir, fatty acid oxidation is markedly decreased, showing that the inhibition takes place *in vivo*. Methyl palmoxirate lowers blood glucose in guinea-pigs, rats,



Methyl Palmoxirate (61)



Clomoxir (62)



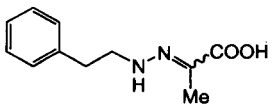
Etomoxir (63)

streptozotocin-diabetic rats, db/db mice and dogs [307], while *rac*-etomoxir was shown to be active in streptozotocin-diabetic rats and db/db mice, lowering cholesterol and triglycerides as well [306]. In IDDM patients, methyl palmoxirate significantly reduced ketonuria and plasma glucose concentrations [310]. Etomoxir, in NIDDM patients, lowers blood glucose, ketone bodies, triglycerides and cholesterol. A euglycaemic clamp on

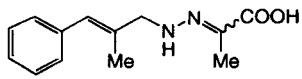
NIDDM patients shows that the main mechanism of glucose lowering is inhibition of glucose output; however, some increase of glucose uptake takes place as well [311, 312].

Carnitine acylcarnitine translocase inhibitors

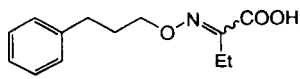
A series of hydrazonopropionic acids shows hypoglycaemic activity in fasted guinea-pigs but is less effective in fed animals [313, 314]. The prototypes 2-(phenethylhydrazono)propionate (PEHP) (64) and 2-(3-methylcinnamylhydrazonopropionate (MCHP) (65) also lower levels of cholesterol and ketone bodies concentrations. The analogue BM 13.677 (66) lowers blood glucose in fasted guinea-pigs after a single dose [315] but has less effect on cholesterol or ketone bodies [314]. MCHP and BM 13.677 inhibit hepatic gluconeogenesis in guinea-pig perfused liver [314, 315], but unlike the glycidic acids, they inhibit the enzyme carnitine-acylcarnitine translocase (K_i = 0.65 mM for MCHP) [316, 317]. BM 13.677 appears to possess extrahepatic effects as well and is reported to be in clinical trials [314].



PEHP (64)



MCHP (65)

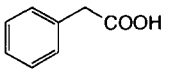


BM 13.677 (66)

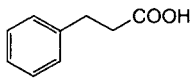
PYRUVATE CARBOXYLASE INHIBITORS

Pyruvate carboxylase is an important enzyme in the gluconeogenic pathway converting pyruvate and bicarbonate into oxaloacetate. AcCoA is required for allosteric activation of the enzyme, and in the diabetic state where fatty acid oxidation is elevated, the high concentrations of AcCoA result in significant activation and gluconeogenesis overactivity. Phenylalkanoic

acids have been shown to block the enzyme and to inhibit gluconeogenesis in the rat isolated perfused liver [318]. *In vivo* studies with phenylacetic acid (67) in db/db mice showed that the compound lowers glucose output [318], while hydrocinnamic acid (68) in streptozotocin-treated and normal rats lowers fasting plasma glucose by suppressing gluconeogenesis, as determined by a euglycaemic clamp [319].



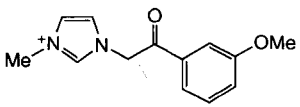
Phenylacetic Acid (67)



Hydrocinnamic acid (68)

PHENACYLIMIDAZOLIUM SALTS

A series of phenacylimidazolium salts has been found to possess *in vivo* hypoglycaemic activity, lowering blood glucose of yellow diabetic mice 2 h after 100 mg/kg administration [320, 321]. Mechanistic studies with one of the most potent compounds, proglycosyn (LY177507) (69), revealed that these compounds stabilize glycogen stores by activating glycogen synthase and inactivating glycogen phosphorylase [322]. Evidence has shown that this is accomplished via stimulation of a phosphoprotein phosphatase which dephosphorylates glycogen synthase and phosphorylase, although the concentrations of proglycosyn required (*ca.* 5mM) are quite high [323].



Proglycosyn (69)

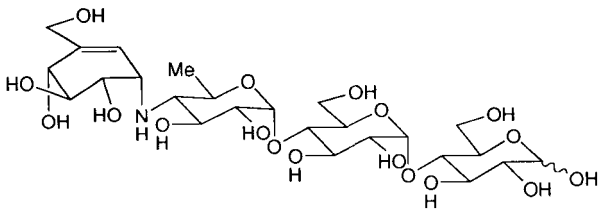
COMPOUNDS ACTING IN THE GUT

α-GLUCOSIDASE INHIBITORS

The absorption of simple and complex carbohydrates from the intestine is mediated by a family of enzymes collectively named α-glucosidases which hydrolyze the oligo- or poly-saccharides to monosaccharides for transport into the blood stream. Inhibition of these enzymes is expected to delay the absorption of carbohydrates and reduce the postprandial glucose rise.

Acarbose

Acarbose (70), a pseudotetrasaccharide containing a carbocyclic ring, was isolated from microbial sources and was the first α-glucosidase inhibitor to be developed. It is now approved in several countries. Acarbose is a reversible competitive inhibitor of glucoamylase and sucrase and acts at the intestinal brush-border. Only 1 to 2% of the drug is absorbed [324]. Acarbose decreases the postprandial glucose rise in healthy volunteers and NIDDM patients when given with each meal. The results of large scale clinical trials have been reviewed recently [325] and more recent studies have found similar results [326, 327]. Acarbose is most effective when given in conjunction with a starchy high-fibre diet [328] and is beneficial in patients poorly controlled by diet alone or by sulphonylureas [329, 330]. Acarbose lowers insulin levels in normal volunteers and has no or little effect on the insulin levels of NIDDM patients. Triglycerides levels are decreased upon treatment, while cholesterol levels are unchanged [324, 330]. The major side-effects are flatulence, abdominal bloating and diarrhoea [331]; however, in a 24-week study the frequency and intensity of these side-effects diminished with time so that at the end of the 24 weeks only 32% of the patients registered weak to moderate complaints and no compliance problem was observed [329]. No hypoglycaemic reaction has been observed. In IDDM patients acarbose lowers postprandial glucose levels and/or insulin requirement and could be used as adjunct therapy to insulin [332, 333].

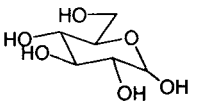


Acarbose (70)

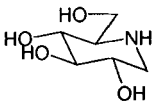
Miglitol

Miglitol (73) is the most potent in a series of derivatives of the natural product moranoline (1-deoxynojirimycin) (72), which appear to closely mimic glucose (71), and which inhibit α-glucoamylase and sucrase [334]. Unlike acarbose, miglitol is almost completely absorbed, possibly via the glucose transporter mechanism, and is excreted through the kidney, a

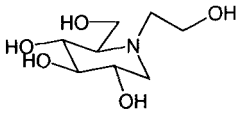
potential advantage. The overall clinical efficacy and side-effect profile are similar to those of acarbose [324, 328]: miglitol lowers the postprandial hyperglycaemia in healthy subjects [335–338] and NIDDM patients [339–344]. A slight improvement in fasting plasma glucose was observed after six months of treatment [344]. Insulin levels were decreased after meals [340, 341] and no effect on cholesterol was detected. In IDDM patients miglitol in conjunction with insulin lowers postprandial glucose more than insulin alone and reduces the insulin requirement [346-350]. Phase III studies are reported to be under way with this agent [324].



D-Glucose (71)



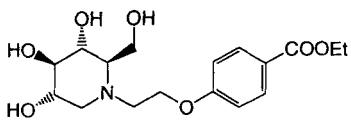
Moranoline (72)



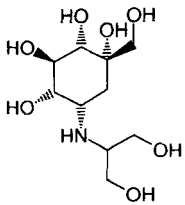
Miglitol 73)

Other α-glucosidase inhibitors

Emiglitate (74) is a longer-acting, more potent nojirimycin derivative which also lowers postprandial glycaemia in healthy subjects and in NIDDM and IDDM patients. However, glycaemic control was not as effective as that seen with miglitol and gastrointestinal side-effects were more common, so that the compound is no longer in development [324]. Voglibose (75) (AO-128) is the preferred compound in a series of valiolamine derivatives [351]. Voglibose improves glucose tolerance in obese subjects after sucrose or meal loading [352]. Castanospermine (76), a compound also investigated

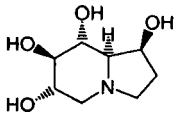


Emiglitate (74)



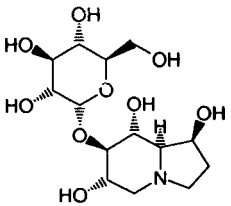
Voglibose (75)

for its anti-HIV activity [353], is a potent competitive sucrase inhibitor, functioning as a transition state analogue [354]. Castanospermine prevents the hyperglycaemic response to an oral glucose load at doses below 1 mg/kg in normal and streptozotocin-diabetic rats. Its prolonged duration of action

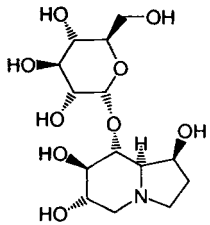


Castanospermine (76)

(over 4 hours as compared to less than 1 hour for acarbose) is attributed to its tight binding to the enzyme [355]. Glucoside analogues of castanospermine have been prepared in order to find compounds more selective toward intestinal glucosidases vs. lysosomal α-glucosidase (an enzyme responsible for the breakdown of glycogen to glucose). Whereas castanospermine displays a 10-fold selectivity, 7-*O*-α-D-glucopyranosyl castanospermine (77)and 8-*O*-α-D-glucopyranosyl castanospermine (78) achieved a 1000-

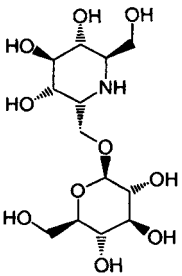


7-*O*-α-D-Glucopyranosyl-castanospermine (77)

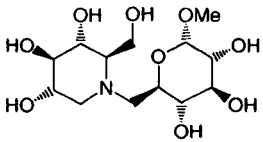


8-*O*-α-D-Glucopyranosyl-castanospermine (78)

fold selectivity [356]. MDL 25,637 (79), a hydrolytically stable sucrose mimic, also functions as a transition state analogue and inhibits the hyperglycaemic response to an oral sucrose or starch load in mice or rats [357]. In a single-dose study in NIDDM patients, MDL 25,637 at 60 mg/kg was effective at lowering postprandial and glucose peaks [358]. Camiglibose

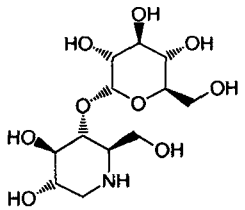


MDL-25,637 (79)



Camiglibose (80)

(MDL 73,945) (80) is a potent, long-duration, nearly irreversible inhibitor which was effective at low doses (0.3–3 mg/kg) at lowering the glycaemic response to a sucrose or starch load in both rats and monkeys. Camiglibose is more selective than castanospermine toward intestinal glucosidases [359]. New deoxynojirimycin analogues such as 4-*O*-α-D-glucopyranosylmoranoline (81) have recently been disclosed that have potent hypoglycaemic activity in the starch-loaded dogs [360].



4-*O*-α-*D*-Glucopyranosylmoranoline (81)

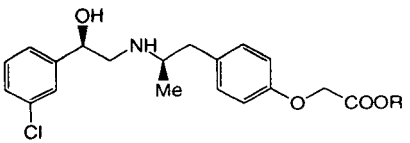
COMPOUNDS ACTING IN FAT: β-AGONISTS

β-Agonists are being developed primarily as antiobesity agents. Although they are designed to increase metabolism in fat, it has been suggested that their hypoglycaemic activity may be a result of effects on insulin action in the muscle. The beneficial effect of exercise for weight reduction has long been believed to be mediated by noradrenaline via the adrenergic receptor. Thus, treatment of healthy volunteers with the β_2-agonist terbutaline stimulates insulin-mediated glucose metabolism [361]. The subtype of the β-receptor associated with thermogenesis in rodents brown fat has been shown to be atypical, that is, neither β_1 nor β_2 and has been classified as β_3 [362–364]. If this receptor subtype also mediates thermogenesis in man, β_3-selective compounds are expected to stimulate thermogenesis without the side-effects usually associated with β_2-agonists, particularly tremors. It was soon found that the prototypical β_3-agonist BRL 26830, when administered to rodents, selectively activates β-receptors in brown adipose tissue, leading to heat production and weight loss, without any change in food intake [365]. BRL 26830 also promotes weight loss in man [365], although tremor was a frequent side-effect, presumably because of residual β_2 stimulation [366, 367]. While BRL 26830 improved glycaemic control in obese or diabetic mice [368, 369], no change in fasting plasma glucose was observed in man after 18 weeks of treatment [365]. However, in a separate study an

improvement in insulin sensitivity was observed in obese subjects after 3 weeks administration of the drug [370]. In mice, the hypoglycaemic effect was associated with a normalization of the glucose transporter (Glut 4) [368] and the insulin receptor [371] numbers in brown adipose tissue, suggesting that this tissue may play a role in glucose regulation in rodents. However, some evidence has been presented to show that some of these hypoglycaemic effects may be mediated by β_3 receptors in the muscle [372]. The recent cloning and expression of the β_3 receptor [373] has made it possible to obtain compounds with high subtype selectivity.

BRL 35135

BRL 35135 (82) is a racemic ester which is rapidly hydrolyzed *in vivo* to its corresponding carboxylic acid, BRL 37344 (83). *In vitro*, BRL 37344 displays a 400-fold and a 21-fold selectivity for the β_3 receptor vs. the β_1 and β_2 subtypes, respectively. In animal models of NIDDM, BRL 35135 upon acute administration increases blood glucose levels *via* an increase in hepatic glucose output. When chronically administered, BRL 35135 improves glucose tolerance and lowers fasting plasma glucose and insulin levels without affecting body weight or body composition [374]. In obese non-diabetic and obese NIDDM patients, 10-day treatment results in an improvement in glucose tolerance independent of body weight and without any change in fasting plasma glucose or insulin levels. A euglycaemic clamp study showed that the improvement was due to an increase in glucose disposal. Mild tremors were observed in some patients [374, 375].

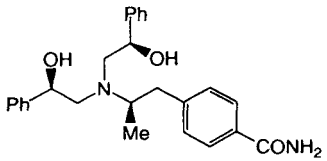


R = Me BRL 35135 (82)
R = H BRL 37344 (83)

OTHER β-AGONISTS

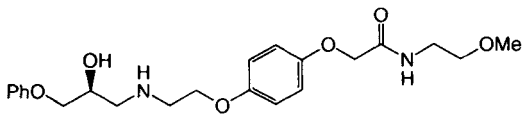
Ro 16-8714 (84) is a tertiary amine also developed as an antiobesity agent. It normalizes blood glucose in ob/ob mice [376] and in streptozotocin-diabetic rats [377] by increasing glucose oxidation. A euglycaemic clamp in fa/fa rats determined that the compound increases both basal and insulin-stimulated glucose utilization in brown adipose fat, the tissue in

which thermogenesis predominantly takes place in rats. This effect is mediated by an increase in the number of insulin-sensitive glucose transporters (Glut4) [378]. In healthy volunteers, a single dose produced a potent thermogenic effect but no hypoglycaemic effect and some tachycardia was observed at the high dose. The latter effect should limit the usefulness of this agent and probably reflects an insufficient β_3/β_1 selectivity [379].



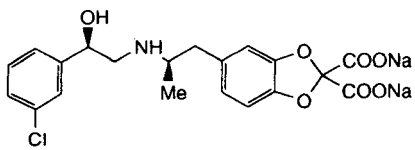
Ro 16-8714 (84)

ICI has investigated a series of phenoxypropanolamines as selective β_3 agonists [380,381]. The leading antiobesity compound, ICI D7114 (85), lowers fasting plasma glucose levels and improves oral glucose tolerance in obese Zucker rats [382].



ICI D7114 (85)

Modifications of the oxyacetic acid portions of BRL 37344 (83) has led Lederle researchers to a series of benzodioxole dicarboxylates. CL 316,243 (86) is an extremely potent agonist at the β_3 receptor and is devoid of significant activity at the β_1 or β_2 receptors. Treatment of ob/ob mice with this compound for 1 week normalized plasma glucose levels to that of untreated lean litter-mates and the euglycaemia was maintained for the next 6 weeks of treatment [383].

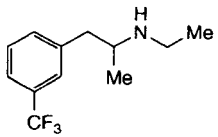


CL 316,243 (86)

The utility of these drugs in man as hypoglycaemic agents remains to be established, since the brown adipose tissue which plays an important role in rodent thermogenesis may not be of paramount importance in man. Selectivity across the receptor subtypes is also likely to be essential for success.

CENTRALLY ACTIVE AGENTS

This section describes compounds developed as CNS agents for the treatment of obesity or depression for which independent hypoglycaemic activity has been observed. Whether or not this secondary effect is also centrally mediated is unclear. The first of these agents to be identified was fenfluramine (87), a serotonin agonist anorectic agent developed in the early 1960s. Its antidiabetic activity has been studied in animals and man and has been recently reviewed [384, 385].

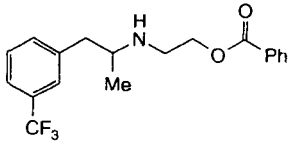


Fenfluramine (87)

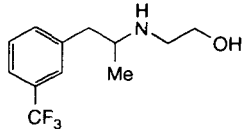
BENFLUOREX

Benfluorex (88) is a benzoyloxy analogue of fenfluramine which is rapidly hydrolyzed *in vivo* to the alcohol S422 (89). It is used as a hypolipidaemic agent and has weight reducing and hypoglycaemic properties as well. Benfluorex lowers the levels of insulin in hyperinsulinaemic models of NIDDM and the levels of fasting glucose in hyperglycaemic animals. This effect can be observed under conditions where no weight loss is observed, so that a direct action on glucose utilization or production has been proposed [384]. In rats made diabetic at birth with streptozotocin, a model of NIDDM, a euglycaemic clamp study showed that benfluorex can significantly reduce hepatic glucose output and slightly enhance glucose utilization, affording an overall improvement of insulin resistance, while body weight remained unchanged [386]. Under acute administration of the drug or its metabolite in normal rats, fasting plasma glucose remained

unchanged while glucose tolerance was improved. The improvement could be explained by an increase in glucose oxidation in muscle [387].



Benfluorex (88)

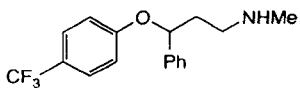


S422 (89)

In NIDDM patients, benfluorex treatment improves glucose tolerance and lowers blood glucose under conditions which do not affect body weight [388–390]. By the euglycaemic clamp technique, improvements both in peripheral glucose utilization [390-392] and hepatic glucose output [391] have been demonstrated. Thus overall benfluorex increases insulin sensitivity.

FLUOXETINE

Fluoxetine (90), a serotonin reuptake inhibitor antidepressant, is being developed as an antiobesity agent, and may possess antidiabetic activity as well. Treatment of NIDDM patients with fluoxetine for 8 to 12 weeks induces weight loss and a drop in the fasting plasma glucose level [393–395]. A euglycaemic clamp showed that peripheral glucose utilization is improved in NIDDM patients after 2 weeks fluoxetine therapy and that this effect is independent of its weight reducing property [396].



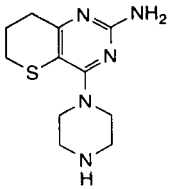
Fluoxetine (90)

MISCELLANEOUS COMPOUNDS

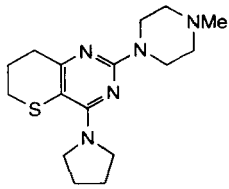
This section gathers hypoglycaemics whose mode of action or target tissue are unreported or unidentified.

THIOPYRANOPYRIMIDINES

MTP-1403 (91) and MTP-1307 (92) are able to improve glucose tolerance in normal rats and in insulin-resistant mice and lower fasting plasma glucose in rats but not in insulin-deficient diabetic animals. In rats, the hypoglycaemic effect is accompanied by an increase in insulin levels, more pronounced with MTP-1307 than with MTP-1403. The mechanism of action is unknown. *In vitro* MTP-1307 inhibits gluconeogenesis in hepatocytes and glucose oxidation in adipose tissue, at 0.1 to 3 mM [397].



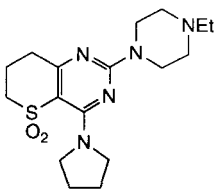
MTP-1403 (91)



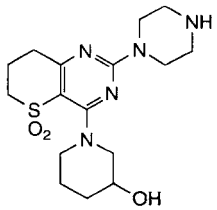
MTP-1307 (92)

MTP-3115 (93), a close derivative of MTP-1307 (92), also improves glucose tolerance in normal and diabetic mice, but unlike the two previous compounds MTP-3115 also lowers blood glucose levels in fed and fasted animals, both normal and insulin-resistant. Since insulin secretagogues do not normally have an effect in insulin-resistant models, MTP-3115 is likely to exert its hypoglycaemic effect by a different mechanism [398], although an insulin releasing effect has been reported [399].

MTP-3631 (94) has a similar profile as MTP-3115; however, it can significantly lower plasma glucose levels in normal rats or ob/ob mice after only 1 hour post-administration. The effect is not accompanied by any insulin release, so that this compound probably acts by a different mechanism from the other thiopyranopyrimidines [399]. The rapid action of MTP-3631 is quite unique among compounds improving insulin sensitivity.



MTP-3115 (93)

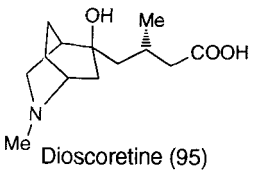


MTP-3631 (94)

More data on these compounds and their mechanism is needed in order to fully evaluate their potential usefulness as antidiabetic agents.

DIOSCORETINE

Dioscoretine (95) is an alkaloid isolated from the tuber of *Dioscorea dumetorum*, a plant used in traditional medicine which contains both hypoglycaemic and hyperglycaemic extracts. In fasted rats, dioscoretine lowers fasting plasma glucose with a maximum reduction after 4 hours. The mechanism of action is unknown [400].



Dioscoretine (95)

CONCLUSION

Research on new therapy for the treatment of IDDM has focused on the discovery of new insulin analogues, so that it is possible to envision a future where a spectrum of insulins will be available for improved 24 h glycemic control. These potential advances will be complemented by new techniques of delivery or new methods for islet transplantation (not reviewed here). After a long period of stagnation since the introduction of sulphonylureas and biguanides for the treatment of NIDDM, compounds with new molecular modes of action are starting to emerge. It is likely that the heterogeneity of the disorder will necessitate a range of available therapies, so that multiple treatment options are available for glycaemic control in order to avoid the severe complications of the disease.

ACKNOWLEDGEMENTS

I wish to thank Drs. E.R. Larson and D.A. Clark for their critical reading of the manuscript and their many useful suggestions.

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Progress in Medicinal Chemistry – Vol. 31, edited by G.P. Ellis and D.K. Luscombe

2 Inhibitors of Human Leukocyte Elastase

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INTRODUCTION

Human leukocyte elastase (HLE; EC 3.4.21.37; also known as human neutrophil elastase, HNE) [l} is a strongly basic glycoprotein which is produced by polymorphonuclear leukocytes (neutrophils) and is released from their azurophilic granules [2]. HLE exists as at least four distinct isozymes, which range in molecular weight from 24 [3] to 30 kDa [4] and appear to differ only in carbohydrate content [5]. Furthermore, human sputum elastase (HSE), which is isolated from purulent sputum as at least five distinct isozymes [6], is both immunologically and catalytically indistinguishable from HLE [7] and is believed to be identical to it.

HLE is a member of the chymotrypsin superfamily of serine proteinases. Each of the enzymes in this group, which also includes α-chymotrypsin (chymo) [8] and trypsin, is capable of degrading a wide variety of proteins. These enzymes are involved in normal physiology as part of the homeostatic regulation of biological systems, and their activity is (normally) regulated by compartmentalization or by endogenous anti-proteinases. HLE, which is potentially one of the most destructive enzymes, is inhibited by both plasma circulating and tissue localized anti-proteinases.

The primary role of intracellular HLE appears to be the degradation of foreign proteins ingested by leukocytes during phagocytosis, whereas the main target for extracellular elastase appears to be elastin [9]. Elastin [10] is the primary elastic component of the lungs, blood vessels and other organs, and is degraded by HLE in a process called elastolysis.

HLE demonstrates a vast array of biologic activities besides the degradation of elastin. A partial, and by no means inclusive, list of some of these activities includes: degradation of fibronectin [11], laminin [12], collagen and proteoglycans [13], and immunoglobulins (IgM, IgA and secretory IgA) [14]; the activation and hydrolysis of complement components and neutrophil complement receptors [15]; stimulation of mucus secretion and induction of mucous gland hypertrophy [16]; and decreases in ciliary beat frequency and mucociliary clearance [17].

The promiscuous nature of HLE and the wide variety of biological activities expressed by it has led to the hypothesis that any imbalance in the delicate equilibrium between HLE and its endogenous inhibitors may result

in, or exacerbate, several pathological states [18]. Such an imbalance could result either from increased release of the enzyme and/or reduced levels of endogenous inhibitors. The fact that degradation of connective tissues is evident in some diseases provides circumstantial evidence to support this hypothesis.

In particular, excessive proteolysis of elastin by HLE has been implicated in pulmonary emphysema [19]. In this case, the imbalance appears to result from reduced levels of active extracellular alpha$_1$-proteinase inhibitor (α_1-PI), the primary plasma inhibitor of HLE. This decrease is caused either by a genetic disorder (PiZZ phenotype individuals) or by reduction in the elastase inhibitory capacity (EIC) of α_1-PI due to its oxidative inactivation by tobacco smoke [20]. The detailed evidence supporting the potential role of elastase in the development of emphysema has been extensively reviewed [21] and will not be repeated here. The fact that HLE is also a potent secretagogue [22] may play a role in several disease states, including cystic fibrosis [23], chronic bronchitis [24], and acute respiratory distress syndrome (ARDS) [25]. The mechanism of the secretagogue activity is not known, but, since the HLE-induced secretion can be blocked by specific HLE inhibitors, it appears to require catalytic activity by the enzyme [26].

One of the possible approaches for restoring the enzyme-inhibitor balance and thereby treating diseases such as emphysema is to supplement the body's level of endogenous inhibitor. Because of this perceived potential utility for HLE inhibitors, a great deal of effort has been expended over the past decade exploring several classes of inhibitors, including natural high-molecular-weight proteinaceous inhibitors, recombinant analogues of these compounds, and low-molecular-weight synthetic inhibitors. The focus of this review will be on compounds which have been evaluated both *in vitro* and *in vivo* and which have been considered as potential clinical candidates.

CATALYTIC MECHANISM

Understanding how HLE inhibitors work and/or designing new inhibitors requires a model of HLE's active-site and an understanding of its mechanism of action. All serine proteinases share a similar catalytic region and mechanism of action but differ in several amino acids in the extended substrate-binding region. These changes are responsible for the specificity differences between HLE and other serine proteinases. In some cases analysis of the enzyme–inhibitor interactions has only been carried out with other related enzymes, and those results are referenced as appropriate. One closely related enzyme, porcine pancreatic elastase (PPE, EC 3.4.21.36) has

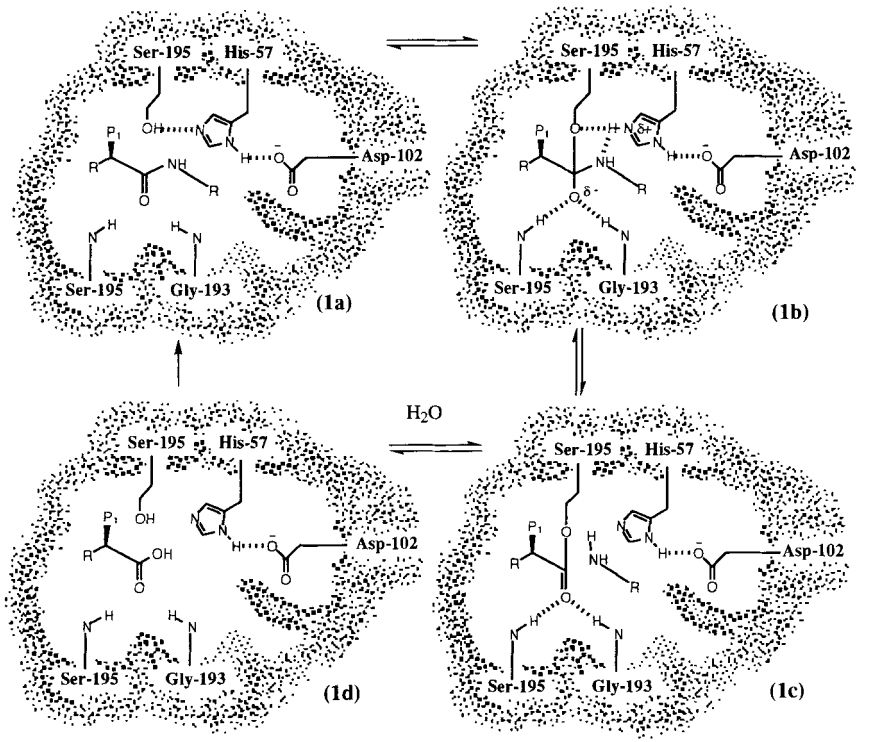


Figure 2.1. The proteolytic mechanism for serine proteinases [240].

been used for many of the mechanistic studies on elastases. It has about 40% primary sequence homology with HLE and has been shown by X-ray crystallographic studies to have a similar active-site region to HLE [27].

Most serine proteinase inhibitors interact with both regions of the active-site: (1) the catalytic site and (2) the extended substrate-binding sites. This double interaction is especially prevalent for those inhibitors which are specific, that is, selective for one enzyme. The catalytic site is generally viewed as being composed of a catalytic triad, made up of Ser-195 [28], His-57 and Asp-102 (see *Figure 2.1*). At one time, it was widely accepted that catalysis was due to a 'charge-relay system' established by these residues, but this hypothesis now seems unlikely [29].

It is now felt that the catalytic mechanism for serine proteinases involves formation of a Michaelis complex (la) followed by attack of the active-site serine hydroxyl on the carbonyl group of the substrate's scissile amide bond

(P_1-P_1' bond) [30] with assistance by the imidazole of His-57 and the carboxylate of Asp-102. This nucleophilic attack of the serine OH forms a high-energy tetrahedral adduct (a 'transition-state intermediate') which is stabilized by hydrogen bonds between the scissile amide oxygen and the NH groups of both Gly-193 and Ser-195 (1b). These two latter residues demark the 'oxyanion hole' [31]. Theory predicts that the enzyme–substrate binding in the actual transition-state complex will be much stronger than that in the initial Michaelis complex and that enzymatic catalysis is a direct result of this feature [32, 33]. Therefore, compounds which can form a 'transition-state like' complex with the enzyme could lead to especially potent inhibitors [33].

Although the actual series of proton transfers is not proven, it appears that the hydrogen atom that was originally on the Oγ of Ser-195 ends up on the amine nitrogen atom of the hydrolysis product. This process goes through a transient species which has anionic character at the oxygen of the scissile amide. Collapse of the tetrahedral intermediate, with cleavage of the amide bond, results in release of the free amine product and formation of an acyl-enzyme intermediate (*Figure 2.1c*).This acyl-enzyme undergoes aqueous hydrolysis, also believed to be catalyzed by His-57, to afford the active enzyme and the product peptide acid (*Figure 2.1d*). The potency of inhibitors specifically designed to mimic the theoretical transition-state for amide bond cleavage, along with X-ray crystallographic confirmation of the predicted binding mode of these inhibitors, provides strong evidence in favour of this catalytic mechanism.

Many serine proteinase inhibitors are active due to their ability to form a covalent link to Ser-195 of the enzyme. However, several proteinaceous compounds which are very potent serine proteinase inhibitors ($K_i < 10^{-9}$M) bind equally well to the parent enzyme and to the corresponding anhydroenzyme, in which the active-site serine has been replaced with dehydroalanine, proving that, with enough contact points, a covalent link to Ser-195 is not required for good activity [see section on proteinaceous inhibitors].

In general, the principal determinants of the specificity of a proteinase for a peptidic substrate/inhibitor are the S_1 subsite and P_1 residue, respectively. Variation of a residue at these locations in either the enzyme or the inhibitor may dramatically change specificity. However, in serine proteinases, secondary binding interactions from the extended binding domain on either side of the catalytic site (e.g. S_4, S_3, S_2, S_1' and S_2') may play a significant role in determining the overall binding of a substrate or inhibitor to the enzyme. For elastases, these secondary interactions are particularly important, because their S_1-subsites usually will not accommodate large or polar amino

acid residues. As examples of this specificity, those peptides which contain Phe, Arg, or Lys residues in the middle of a chain and which are constrained such that these amino acids must be the P_1-residue will be inactive either as substrates or inhibitors of elastases. This is particularly true for PPE which prefers to accommodate Gly or Ala as the P_1-residue [27]. The steric restrictions for HLE are less stringent and it will accommodate P_1-residues such as Met, Val, and Ile [34]. In contrast, some peptides can be cleaved by elastases at their N-terminal amino acid amide bond even if that residue is Phe, Arg, or Lys.

The relatively small size of the S_1 subsite in elastases means that there is no possibility for a dominant P_1-S_1 binding interaction, such as occurs with the recognition of a P_1-Arg by trypsin [35]. Therefore, for elastases, the extended binding interactions become relatively more important. With HLE, recognition of specific amino acid residues has been demonstrated to extend to at least S_5 [36] and to S_3' [37]. The cumulative binding forces over this octapeptide region may be quite large and can allow large peptides to retain good levels of activity without establishing a covalent link. In contrast, low-molecular-weight inhibitors require the formation of a covalent adduct to attain significant potency.

DISCUSSION OF ASSAYS

Characterization of the naturally occurring and the synthetic inhibitors has been accomplished using various assays. A brief description of the more common kinetic parameters and assays follows.

Measurement of the *in vitro* efficacy of compounds as substrates is usually deduced by comparison of their k_{cat}/K_m ratios where k_{cat} is the first-order rate constant for product formation and K_m is the Michaelis equilibrium constant [38]. For those compounds which are classical, reversible inhibitors, K_i, the dissociation (or inhibition) equilibrium constant, and k_{on} (k_{assoc}), the rate constant for enzyme inhibition, are the most commonly reported kinetic values. These values may be measured while using either a high-molecular-weight natural substrate or a low-molecular-weight synthetic substrate. For alternate-substrate inhibitors, that is, compounds which form a stable complex (an acyl-enzyme?) that dissociates to enzyme and intact inhibitor or to enzyme and an altered form of the inhibitor, the usually reported value is K_i^*, the apparent K_i. For compounds which irreversibly inactivate the enzyme, the kinetics are usually measured under conditions such that the initial enzyme concentration $[E]_o$ is much lower than the inhibitor concentration [I] which in turn is much lower than the K_i. Under these conditions the commonly reported value is $k_{obs}/[I]$, the apparent

pseudo-first-order rate constant for inactivation divided by the concentration of inhibitor. If the kinetics of an irreversible inactivator are measured as a function of [I], at concentration ranges which will have an effect on k_{obs}/[I], the kinetics are usually reported as k_2/K_i, where k_2 is the first-order rate constant for formation of the inactivated enzyme. Since small changes in the *in vitro* assay conditions (for example, substrate, concentration, pH, etc.) can change the measured kinetic values [39], comparison of relative K_i and k_{assoc} values will mostly be limited to those cases where the same researchers have reported a series of compounds.

The mere fact that a compound inactivates HLE *in vitro* is not in itself a guarantee for a physiological role. Bieth [40] has proposed that kinetic parameters may be used to predict the *in vivo* efficacy of proteinase inhibitors via calculation of a half-life for inactivation ($t_{1/2inact}$). This value is determined according to the following formula: $t_{1/2inact} \cong 1/[k_{assoc}][I]$. The size of $t_{1/2inact}$ indicates whether the rate of inhibition is fast enough to be physiologically significant, with a predicted limit on the order of milliseconds [41]. Although this proposal has been accepted and widely quoted with respect to the proteinaceous inhibitors, its relevance to the low-molecular-weight inhibitors is in question. This is due to the fact that some potent but slow-binding inhibitors, e.g. ICI 200,880 which has a k_{on} value of 9×10^4 $M^{-1}s^{-1}$ towards HLE, have been shown to be efficacious inhibitors of HLE in *in vivo* models [42].

In vivo efficacy of elastase inhibitors has generally been studied in animal models of lung injury or inflammation (shock). Most of the lung injury studies have been in hamster or mouse models. In general, either HLE or PPE is instilled intratracheally, which leads both to acute lung injury and to induction of a slowly progressive, destructive, emphysema-like, lesion of the lung. The severity of the acute injury is usually determined by measuring the extent of haemorrhage. This is proportional to the amount of haemoglobin measured (the acute haemorrhagic assay or AHA model) [43] or by counting the number of specific blood cells that are recovered (the acute lung injury or ALI model) [42], upon lung lavage. An emphysema-like state (EMP model) [44] is determined over a 6–8 week period by measurement of pulmonary function parameters and/or morphological examination. The compound may be administered orally (p.o.), intraperitoneally (i.p.), or intratracheally (i.t.). In the latter case, the compound may be administered admixed with the HLE/PPE, in addition to the more common regimens of dosing pre- or post-enzyme. Sepsis in guinea-pigs has been utilized as an *in vivo* inflammation model.

It is important to note that the 'effectiveness' in the *in vivo* models for a given dose of inhibitor will vary by the route of administration, the relative

timing of inhibitor administration, and the dose of enzyme used to produce the insult. Therefore, comparisons of different series of inhibitors across different studies will not be drawn. Also, statistically significant activity will usually be reported as being determined for a specified route of administration and at a given dose of inhibitor but without always specifying either the relative time of inhibitor dosing or the dose of elastase used as an insult.

PROTEINACEOUS INHIBITORS

INTRODUCTION

There are many high-molecular-weight, polypeptide, elastase inhibitors which have been isolated from animal or plant sources. Most of these, the non-human proteins, would probably induce an immunogenic response and are not suitable for clinical development. However, a subset of these inhibitors, predominantly human in origin, is being explored as a source for clinically-useful elastase inhibitors. Each of the human compounds is found in a specific location, which probably is its primary site for inhibitory action. The physical properties of many of these natural inhibitors have been reported (see *Table 2.1*)[45–51]. Due to their size and other physical properties, only intravenous or topical formulations of the proteinaceous inhibitors have been considered for clinical use. The pharmacological studies have included natural inhibitors, recombinant variants (i.e. peptides with identical sequences to the natural inhibitors but not necessarily the same glycosylation) and recombinant mutants (peptides with unnatural sequences) [52].

α_1-PROTEINASE INHIBITOR AND MECHANISMS OF ACTION OF PROTEINACEOUS INHIBITORS

The two main plasma inhibitors of HLE are α_1-proteinase inhibitor (α_1-PI) and α_2-macroglobulin (α_2-M). Comparison of the $t_{1/2inact}$ calculated for α_1-PI and α_2-M (*Table 2.1*) indicates that α_1-PI should be considered the primary plasma inhibitor of HLE. This assumption is corroborated by the finding that when mixtures of human serum and labelled HLE are analyzed they show that 92% of the HLE is complexed to α_1-PI and only 8% is complexed to α_2-M [53].

α_1-PI (also known as α_1-antitrypsin, α_1-AT) [54] is an extremely potent and fast acting inhibitor of HLE [45, 46]. It is a glycopeptide, composed of

Table 2.1. NATURAL INHIBITORS: PHYSICAL PROPERTIES AND KINETIC VALUES

Inhibitor	*MW*	*Kinetic parameters with HLE*				
		k_{asoc} $(M^{-1}s^{-1})$	k_{diss} $s^{-1} \times 10^3$	K_i (M^{-1})	$t_{1/2inact}$[a] $(s^{-1} \times 10^{-3})$	*Ref.*
α_1-PI	52,000	6.5×10^7		3.3×10^{-14}	0.6	45,46
SLPI	11,700	6.4×10^6	2.3	3.3×10^{-10}	150[b]	51
Elafin	7,017			6×10^{-10}		49
EIM	42,741					48
α_2-M	725,000	4.1×10^7			4–11[c]	47
Eglin C	8,100	1×10^6	8×10^{-4}	8×10^{-10}	500[d]	50

[a]$t_{1/2inact}$ calculated based on expected physiological concentration in serum.
[b]Based on a concentration of 5μM in secretions.
[c]Calculated [47].
[d]Calculated at a proposed concentration of 2 μM.
[e]Abbreviations used in tables include: Ada = 1-adamantyl; boro-AA-OH = boronic acid analogue of specified amino acid (aa); Cbz = benzyloxycarbonyl; CMK = chloromethylketone; EIM = enzyme inhibitor of monocytes; -NA = *p*-nitroanilide; ND = not determined; NR = not reactive; MeO-Suc = methoxy succinoyl; Met(O) = methionine sulphoxide; Pic = picolinyl; PPE = porcine pancreatic elastase; SLPI = secretory leukocyte proteinase inhibitor; TFMK = trifluoromethylketone; Z = benzyloxycarbonyl.

a single polypeptide chain made up of 394 amino acid residues and three carbohydrate side-chains, which is predominantly synthesized and secreted by hepatocytes. The moderate size of α_1-PI allows it to migrate relatively freely from the circulatory system into tissues, where it forms a 1:1 inhibitor–enzyme complex. This complex readily migrates back into the circulatory system, from which it is rapidly cleared. Although α_1-PI inhibits a wide number of proteinases, kinetic studies show that it most rapidly associates with HLE, implying that HLE is the major target of α_1-PI.

α_1-PI is a member of the serpin (serine proteinase inhibitor) superfamily of inhibitors, which have a 'so-called' unusual mechanism of inhibition. There are at least 10 families of protein inhibitors of serine proteinases [52a], and some, which had been characterized earlier than the serpin family (for example, the Kunitz and Kazal families), were found to have a common mechanism of action [52a]. Their mode of action came to be known as the 'standard mechanism' of proteinase inhibition and is described by the following simplified kinetic scheme (*Equation 2.1*):

Equation 2.1. $E + I \rightleftharpoons E{\cdot}I \rightleftharpoons C \rightleftharpoons E{\cdot}I^* \rightleftharpoons E + I^*$

in which E is the proteinase, I is the virgin inhibitor (reactive site intact), I* is the modified inhibitor (reactive site cleaved), E·I and E·I* are loose Michaelis complexes, and C is the stable reversible complex (believed in some cases to be the tetrahedral adduct).

The inhibitors bind the enzyme extremely tightly, as if they were exceptionally good substrates. However, their rate of hydrolysis is extremely slow and hydrolysis does not proceed to completion. At neutral pH the equilibrium constant between modified inhibitor and virgin inhibitor is approximately one, and the proteinase–inhibitor interaction appears to be fully reversible. It is noteworthy that both the virgin and modified inhibitors bind the proteinase. In part, this is due to the fact that disulphide bridges in the inhibitor stabilize the two peptide chains in I* such that the conformation of the reactive site region in them is similar to that in I. However, the k_{assoc} determined for I* is generally much slower than the k_{assoc} of the virgin inhibitor [52a]. Although the reason for this is not proven it seems likely that the charged nature of the free amino acids in I* interfere with binding to the enzyme.

Bode et al. [3] have determined the X-ray structure of the complex formed by turkey ovomucoid inhibitor (TOMI), an example of a standard mechanism inhibitor, bound to HLE. The structure shows that, although the reactive-site peptide bond in the HLE-TOMI complex is intact, the P_1-carbonyl group is distorted and displays a partial tetrahedral geometry. The distortion appears to be induced by hydrogen bonding of the P_1-carbonyl oxygen to the NH groups of Gly-193 and Ser-195 ('the oxyanion hole') and results in a conformation similar to that expected for a pre-transition-state complex. Further evidence that this distortion is primarily due to the hydrogen bonds between the carbonyl oxygen and the oxyanion hole (and not to addition of the Oγ of Ser-195 to the P_1-carbonyl group) comes from related studies which showed that pancreatic trypsin inhibitor (also a standard mechanism inhibitor) has a similar overall geometry in the complexes formed with either bovine trypsin [55] or bovine anhydrotrypsin [56], even though there is no serine hydroxyl in the latter compound.

In contrast to the protein inhibitors which act by the 'standard mechanism of proteinase inhibition' [52a], α_1-PI reacts only weakly with anhydrochymotrypsin [57] and not at all with anhydrotrypsin [58]. Also, unlike I* (the product of the standard mechanism inhibitors), α_1-PI* (α_1-PI which has been cleaved) is not a proteinase inhibitor.

Upon dissociation of the α_1-PI-HLE complex, induced either by

denaturization or by non-specific proteolysis, only α_1-PI* has been isolated. The enzyme–inhibitor complexes formed from α_1-PI with either human trypsin, chymotrypsin, or elastase are very stable and appear to be irreversible, as each complex inhibits neither of the other two enzymes. This finding implied that the complex might be an acyl-enzyme. In contrast to these studies, it has also been observed that, in the presence of an excess of α_2-M as a trap, the α_1-PI-porcine trypsin complex dissociates very slowly, both forward to give α_1-PI* and backwards to give active α_1-PI [59]. Since the acyl-enzyme could not lead to α_1-PI (see next paragraph), this finding established that for the α_1-PI-porcine trypsin complex, the intermediate could not be the acyl-enzyme.

Although native α_1-PI has not been successfully crystallized and its three-dimensional structure remains speculative, α_1-PI* has been crystallized and its structure analyzed by high resolution X-ray studies [60]. Surprisingly, the two amino acids (Met-358 and Ser-359) which had been joined to make the reactive bond of the inhibitor are separated by 69Å in α_1-PI* [60]. This finding demonstrates that a major reorganization of the three-dimensional structure of the inhibitor has taken place and explains why α_1-PI* is not an inhibitor. As a result of this rearrangement the denaturization temperature of α_1-PI increases from < 60°C to >80°C for α_1-PI* [61]. This increase in thermal stability implies that the peptide has undergone a change from a stressed conformation to a relaxed form. This reorganization is presumed to occur immediately following reactive-bond cleavage and is why an acyl-enzyme complex formed with α_1-PI could not revert to α_1-PI.

In spite of α_1-PI's potency as an inhibitor, elastin binds HLE so tighly that it effectively competes with α_1-PI for binding to HLE. This effect is such that the K_i measured for α_1-PI and for many other inhibitors increases if elastin is used as a substrate instead of a low-molecular-weight synthetic compound [39]. It has also been shown that ~25% of the activity of elastin-bound elastase is resistant to inhibition by α_1-PI [62]. One hypothesis is that α_1-PI may be too large to effectively interact with HLE that is tightly bound to a very large substrate.

Theoretically, all of the proteinaceous inhibitors act by presenting a loop portion of their chains as an idealized, that is, pre-organized for optimal interactions, substrate for elastase. Conformational analysis of the ϕ and ψ angles for inhibitor residues P_1–P_3 and P_1–P_3' for a series of proteinaceous, serine protease inhibitors showed that there is little difference between their free and/or complexed states [63]. In α_1-PI, the loop contains as its elastase reactive center (see *Table 2.2*) a Met-Ser linkage. The importance of the P_1-substituent in α_1-PI for its enzyme specificity characteristics is exempli-

Table 2.2. PROPOSED REACTIVE SITES OF PEPTIDE PROTEINASE INHIBITORS[a]

Inhibitor	P_4	P_3	P_2	P_1	P_1'	P_2'	P_3'	P_4'	P_5'	*Ref.*
α_1-PI	Ala-355	Ile	Pro	Met	Ser	Ile	Pro	Pro	Glu	3
SLPI	Gly-69	Gln	Cys	Leu	Met	Leu	Asn	Pro	Pro	65
Elafin	Leu-20	Arg	Cys	Ala	Met	Leu	Asn	Pro	Pro	49
EIM	Ala-341	Thr	Phe	Cys	Met	Leu	Met	Pro	Glu	48
Eglin C	Pro-42	Val	Thr	Leu	Asp	Leu	Arg	Tyr	Asn	66

[a]See footnote 'e' in *Table 2.1*.

fied by the fact that a natural mutant form, known as the α_1-PI-Pittsburgh variant [64], which varies only by replacement of Met-358 with Arg-358 shows no activity against elastase and excellent activity against thrombin. However, X-ray analysis of HLE bound to other inhibitors implied that an even better fit to HLE might occur if the P_1-Met was replaced by Val [3]. This prompted the query, why would evolution lead to a less than ideal inhibitor?

The answer may lie in the fact that methionine is very sensitive to oxidation, and α_1-PI which has been oxidized at this centre (α_1-PI_{ox}) is both a much less potent ($K_i = 1.5 \times 10^{-10}$ M) and slower acting ($k_{assoc} = 3.1 \times 10^4$ M^{-1} s^{-1}) inhibitor of HLE [45, 46]. This decrease in activity can be rationalized by the fact that oxidation of the methionine sulphur to a sulphoxide increases the size and polarity of the P_1-substituent. The increase in size may directly interfere with fitting this residue into the S_1-subsite of HLE, and the change in polarity may cause a reduction in the net binding energy gained by the transition from a purely aqueous solution to a bound environment.

It appears probable that this sensitivity to oxidation is both part of the homeostatic control mechanism for HLE and a possible basis for some disease processes. At physiological concentrations, the $t_{1/2inact}$ increases from 0.64 ms for α_1-PI to ~1.3 s for α_1-PI_{ox}, and the latter compound is ineffective *in vivo* as an inhibitor of HLE [45]. It is now believed that the oxidative burst of an activated neutrophil deactivates any α_1-PI in close proximity by conversion to α_1-PI_{ox}, thus allowing elastase to remain active. Since the effective range of oxidative radicals is very limited, α_1-PI outside the immediate vicinity of the neutrophil retains its activity and inhibits systemic HLE.

Inactivation of α_1-PI by conversion to α_1-PI_{ox} has been shown to occur *in*

vitro upon exposure to cigarette smoke [67, 20]. This data led to the 'oxidation hypothesis' which attempted to explain the correlation between smoking and emphysema [68]. Additional circumstantial evidence for the link between an HLE/α_1-PI imbalance and emphysema is supplied by the fact that i.t. α_1-PI protects against haemorrhage in the hamster AHA model [43].

As potentially more readily-available alternatives to natural α_1-PI, the supply of which is limited, recombinant α_1-PI (r-α_1-PI) and a mutant form incorporating the previously suggested change of Met-358 to Val-358 (r-α_1-PI_{val}) have been produced and expressed in yeast [69, 70]. In part, the valine mutant was chosen in order to test whether it would confer stability to oxidation. As hypothesized, the r-α_1-PI_{val} form was shown to be resistant to both chemical oxidation [69] and to inactivation by neutrophils [71]. This result indirectly supported the proposal that Met-358 acts as a switch by which neutrophils inactivate α_1-PI. Although both of these recombinant peptides are active as inhibitors of HLE, they are much less thermally stable and show significantly shorter half-lives *in vivo* than the native inhibitor [70]. These differences have been ascribed to the fact that neither of these variants incorporates the carbohydrate side-chains found in native α_1-PI.

α_2-MACROGLOBULIN

α_2-Macroglobulin (α_2-M) is a very effective ($\geq$ 1 molecule of HLE inhibited per molecule of α_2-M) and fast acting plasma inhibitor of HLE [47]. Like α_1-PI, it is a glycoprotein, but, due to its larger size, it does not readily migrate from plasma to tissue spaces. α_2-M appears to work via a unique entrapment mechanism. This process prevents interaction of the entrapped enzyme with large compounds (that is, elastin or α_1-PI) but does not inhibit the entrapped enzyme's ability to interact with low-molecular-weight substrates or inhibitors. Since α_2-M is an essentially non-specific endopeptidase inhibitor (which is active against all four types of endopeptidases: serine, aspartic acid, cysteine and metalloproteinases [47]) it will not be discussed further in this review.

ENZYME INHIBITOR OF MONOCYTES

The enzyme inhibitor of monocytes (EIM), is a proteinaceous inhibitor of HLE which is found in human monocytes, neutrophils, and macrophages [72]. It was recently characterized and was shown to be made up of a single, 379 amino acid chain [48]. So far the detailed kinetic constants for EIM have not been reported nor is there any evidence that EIM is secreted [48]. The

mechanism of action of EIM is presumed to be similar to α_1-PI since it also forms an enzyme–inhibitor complex which is stable to boiling sodium dodecylsulphate solution, but which is susceptible to nucleophilic cleavage [73, 74]. Due to this feature, the fact that there is 30% sequence homology between EIM and α_1-PI, and its Cys-Met reactive site, it has been designated a Cys-serpin [48].

SECRETORY LEUKOCYTE PROTEINASE INHIBITOR

Secretory leukocyte proteinase inhibitor (SLPI) [75-77] is a potent, fast acting, acid-stable, reversible inhibitor of HLE [51]. It is also a potent inhibitor of human cathepsin G ($K_i = 4 \times 10^{-9}$ M), bovine trypsin ($K_i = 8 \times 10^{-9}$ M), and bovine α-chymotrypsin ($K_i = 3 \times 10^{-8}$ M). These kinetic values highlight the fact that SLPI, like the other proteinaceous inhibitors, is not a specific inhibitor of HLE. It has been detected in human serum, bronchial secretions, cervical mucus, seminal fluid and parotid secretions [49]. It is a relatively small polypeptide, made up of a single 107 amino acid chain, which is divided into two domains of similar size and three-dimensional structure [65, 78]. Unlike the serpins, its architecture is rigidly fixed by 8 disulphide bridges, 4 in each domain. Although limited homology exists between residues 72–81 of SLPI and the consensus sequence for the active-site of Kazal inhibitors [78a], due to its size, sequence and 3-dimensional architecture, SLPI appears to be the first example of a new family of protein inhibitors [78b]. X-ray analysis of a complex of SLPI with bovine α-chymotrypsin reveals that the reactive bond (with this enzyme) is between Leu-72 and Met-73.

In contrast to the result obtained with α_1-PI, SLPI was able to inhibit 100% of the elastolytic activity of HLE in the presence of elastin [62]. Several small molecule inhibitors (for example, a chloromethylketone and a beta-lactam) were also less effective than SLPI at blocking elastase in an assay in which elastase is placed on a layer of elastin, that is, elastin as a subjacent substrate [79]. Recombinant SLPI (rSLPI), expressed in *Escherichia coli*, has similar *in vitro* properties to naturally occurring SLPI [80,81,82]. Incubation of SLPI with either stimulated human polymorphonuclear leucocytes (PMNs) [83] or cigarette smoke [67] resulted in a rapid loss of anti-elastase activity, which paralleled that shown by α_1-PI. A related study on rSLPI showed a similar degree of sensitivity to *in vitro* oxidative deactivation as had been observed for α_1-PI [84]. This deactivation was inversely correlated with the degree of methionine oxidation, thereby providing circumstantial evidence that those natural inhibitors which have

an active-site Met (for example, SLPI, EIM, and Elafin) may all be, like α_1-PI, subject to biological regulation by activated neutrophils.

Addition of a low-molecular-weight substrate to the equilibrium mixture formed from equimolar amounts of SLPI and HLE resulted in time-dependent release of product, indicating that enzyme, inhibitor, substrate and their complexes were in true equilibrium [51]. Incubation of rSLPI–HLE complex with α_1-PI resulted in dissociation of rSLPI and formation of an α_1-PI–HLE complex. The fact that the dissociated rSLPI retained its ability to inhibit HLE is further indication that it may be a 'standard mechanism' of action inhibitor [82]. *In vivo*, intratracheally instilled rSLPI is capable of significantly protecting against HLE-induced emphysema in the hamster (EMP model) for pretreatment times of up to 8 h [82].

ELAFIN

There have been reports of two low-molecular-weight inhibitors of HLE isolated from the scales of patients with psoriasis: elafin and psoriatic inhibitor. Elafin is a single chain polypeptide composed of 57 amino acids with a total MW of 7017 which also inhibits PPE with a K_i value of 1×10^{-9} M [49]. It appears to be specific for elastases and is inactive against other related enzymes such as human cathepsin G, bovine α-chymotrypsin, porcine trypsin and human plasmin. Elafin appears to be most closely related to the C-terminal half of SLPI, with which it shares 38% homology. It is the only known human protein which appears to be related to SLPI. Notably, the key amino acids at the P_1'–P_6' positions of the proposed reactive centre and the positions occupied by all eight cysteines in Elafin and the C-terminal domain of SLPI are identical. However, the disulphide bonding order of elafin has not been established, and it is not known whether the overall architecture of the two inhibitors is similar. Recombinant elafin (rElafin) has been prepared and has been shown to have identical *in vitro* properties to elafin [85]. rElafin has also been shown to be effective *in vivo*, following intratracheal administration in the ALI model [85].

Psoriatic inhibitor has a molecular mass of ~9 kDa based on gel permeation, and is not active against PPE [86]. Due to the limited data available, it is not clear whether psoriatic inhibitor and elafin are different peptides or whether variation in isolation techniques have resulted in partial degradation of the native inhibitor to produce these differences. In this regard, it should be noted that when the molecular mass of elafin is estimated by gel permeation it also yields a value of ~9 kDa.

EGLIN C

Although not of human origin, eglin B and eglin C [87], which were isolated from the leech *Hirudo medicinalis*, are potent, competitive, reversible inhibitors of both human elastase and cathepsin G, with K_i values against both enzymes of ~2–3 × 10^{-10} M [50, 87]. Eglin B and eglin C each consist of a single polypeptide chain of 70 amino acids [94]. Eglin B has similar properties to eglin C and differs from it only by having a His-35 substituting for a Tyr-35 amino acid residue [88].

Unlike the previously described proteinaceous inhibitors, eglin C has no sulfur containing residues, and it is remarkably stable to acid, heat, and non-specific proteolysis [89]. The (chymotrypsin) reactive bond of eglin C is between amino acid residues Leu-45 and Asp-46 [90]. This location has also been confirmed by a crystal structure with subtilisin Carlsberg [94]. Indirect confirmation that this is also the reactive bond with HLE is supplied by the fact that replacement of the Leu-45 residue with Arg results in: loss of almost all inhibition of HLE; retention of similar levels of inhibition of subtilisin (a serine proteinase); and excellent inhibition of trypsin [91]. The absence of an oxidizable methionine at either the P_1-or P_1'-positions means that eglin C, unlike α_1-PI or SLPI, is not inactivated by the neutrophil's oxidative burst. Like SLPI, recombinant eglin C (r-Eglin C) has been prepared and shown to have a similar *in vitro* profile to the native inhibitor [80]. Also like SLPI, eglin C completely inhibits the action of HLE on elastin [92]. The *in vivo* efficacy of r-eglin C has been demonstrated in a lipopolysaccharide-induced, porcine endotoxin shock model [93]. Although r-eglin C has been proposed as a potential therapeutic agent, concerns remain over its potential to induce an immunogenic response [94].

SYNTHETIC INHIBITORS

INTRODUCTION

A clinically useful alternative to the high-molecular-weight proteinaceous inhibitors discussed above would be the development of low-molecular-weight, synthetic elastase inhibitors. Such compounds offer several theoretical advantages including: greater enzyme selectivity, potential oral activity, less susceptibility to proteolytic inactivation, decreased risk of immunogenic response, and a greater ability to subtly modify the inhibitor structure as a means of optimizing physical and pharmacokinetic properties for production of viable clinical candidates. Out of the many series of

synthetic HLE inhibitors which have been reported, the emphasis here will be on potent ($K_i < 10^{-6}$ M), reversible inhibitors and fast-acting ($k_{obs}/[I] > 10^3$ $M^{-1}s^{-1}$), irreversible inactivators which have been examined in *in vivo* models.

For ease of discussion, these compounds have been divided into three classes: peptide-based, reversible inhibitors; nonpeptide, acyclic acylating agents; and heterocyclic inhibitors. The first compounds require a short peptide sequence to interact with the extended binding region of HLE. This 'recognition' is critical to the potency and specificity of the peptide-based series, although the mechanistic basis for inhibition is due to other specific interactions with components of the catalytic triad. The acylating agents owe their activity to irreversible covalent attachment to either Ser-195 or His-57. By irreversible we do not mean to imply that the enzyme is permanently inactivated. Rather, these compounds are irreversible inhibitors in the sense that their mechanism of action is such that if the enzyme is reactivated, the inhibitor will not always be recovered intact. An example of this class would be an alternate-substrate inhibitor, that is, a compound which rapidly progresses to an acyl enzyme but whose hydrolysis to free enzyme and either processed or recovered inhibitor is relatively slow. Heterocyclic inhibitors react with one of the residues in the active-site (usually Ser-195) such that the heterocyclic ring is destroyed and the inhibitor becomes covalently linked to the enzyme.

PEPTIDE SUBSTRATES

Many of the peptide backbones used in the low-molecular-weight, peptide inhibitors of HLE are based on the pioneering work of Powers *et al.* and Thompson and Blout. The initial studies focused on small substrates and inhibitors of PPE and HLE [95] (see *Table 2.3*). Thompson and Blout showed that PPE preferred cleaving peptides at Ala residues and that the efficiency of cleavage (e.g. k_{cat}/K_m) increased with chain length, out to at least tetrapeptides [96]. It was also demonstrated that a proline residue was strongly disfavoured at either the P_1- or P_3-residue [97].

Starting with a poly-Ala derivative (3-1) Powers determined that both PPE and HLE strongly preferred a P_2-Pro, e.g. X-Ala-Ala-Pro-Ala-Y where X and Y are non-amino acid end groups [95]. As has been shown for the proteinaceous inhibitors, the ϕ, ψ angles available to a P_2-Pro undoubtedly impart a degree of structural preorganization to the inhibitor, which improves its fit in the enzyme. X-ray crystallographic structures of some peptide-based, low-molecular-weight inhibitors bound to HLE have shown that the P_2-Pro residue imparts a twist in the orientation of the amino acid

Table 2.3. KINETIC CONSTANTS FOR HYDROLYSIS OF SYNTHETIC 4-NITROANILIDES BY HLE[a]

Compound	*Substrate*					k_{cat}/K_m[b] (M^{-1} s^{-1})
	P_5	P_4	P_3	P_2	P_1	
(3-1)		Suc	Ala	Ala	Ala-NA	570
(3-2)	Ac	Ala	Ala	Pro	Val-NA	27,000
(3-3)	MeO-Suc	Ala	Ala	Pro	Val-NA	120,000
(3-4)	MeO-Suc	Ala	Ala	Pro	Met-NA	300
(3-5)	MeO-Suc	Ala	Ala	Pro	Met(O)-NA[c]	NR
(3-6)	MeO-Suc	Ala	Ile	Pro	Met-NA	4,000

[a]See footnote 'e' in *Table 2.1.*
[b]All data from [95].
[c]Both sulphoxide diastereomers were non-reactive.

chain which allows inhibitors or substrates to bind to the enzyme in an extended β-sheet arrangement (see *Figure 2.2*) [98]. The P_1-carbonyl oxygen and NH are H-bonded to the NHs of Ser-195 and Gly-193 and the C=O of Ser-214, respectively. The β-sheet geometry allows for hydrogen bonds between the P_3-carbonyl oxygen and the NH of Val-216 and between the P_3-NH and the Val-216 carbonyl oxygen. These additional H-bonds to the P_3-residue are the most likely explanation for the increase in activity observed for tripeptide inhibitors and substrates over the corresponding dipeptides. Recent molecular dynamic simulation of a Michaelis complex between PPE and a hexapeptide substrate (Thr-Pro-nVal-Leu-Tyr-Thr) shows many of these H-bonds and reveals an additional one between Gln-192 (N_ε–H^1 or N_ε–H^2) and the P_2-Pro (C=O) [99]. This last interaction is not possible in HLE, which has a Phe-192 residue. Powers' *et al.* findings also revealed that while PPE preferred a P_1-Ala residue, HLE preferred a

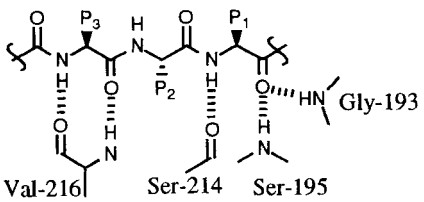


Figure 2.2. Hydrogen-bonding interactions between peptides and HLE [240].

P_1-Val residue, e.g. (3-2) [95]. The discovery that HLE displayed favourable interactions with compounds out to the S_4-S_5 subsites, as shown by substrate (3-3) [95], supported the previously reported data on the importance of the extended binding region in HLE.

The early substrate series had P_1-residues which incorporated a *para*-nitroanilide in place of the normal scissile amide bond. This feature allowed the kinetics of the hydrolyses to be easily followed spectrophotometrically [95]. As a result, these studies focused on the S_1–S_5 subsites. Some of these substrates, for example (3-4)–(3-6), were based on the sequence of the reactive centre of α_1-PI. It was shown that substrate (3-3) which contains a P_1-Val is a better substrate than (3-4) which contains the P_1-Met found in α_1-PI (see *Table 2.2*). As in α_1-PI, oxidation of the P_1-Met resulted in a decrease in enzyme recognition, for example, compare (3-4) and (3-5). One of the compounds (3-3) prepared in the course of this work is an excellent, specific substrate for HLE and also has better aqueous solubility than other related substrates. As a result of this combination of properties, compound (3-3) has been used as the standard low-molecular-weight substrate for much of the kinetic analyses of HLE inhibitors, and its peptide backbone has been incorporated into several low-molecular-weight inhibitors.

Further changes in the P_1–P_5 region, which resulted in improved efficacy for these synthetic substrates, were based on elastin, HLE's natural substrate [36]. This excellent substrate is an insoluble, structural protein, which is primarily composed of hydrophobic amino acid residues. However, it also contains a number of Lys-derived, cross-linked residues, such as desmosine and isodesmosine, that incorporate a positively charged pyridinium ring. In order to model this cross-linking feature, Lys or various amino-protected forms of Lys, were systematically incorporated into the substrate MeO-Suc-Ala-Ala-Pro-Val-NA (4-1). Replacement of any single residue with Lys led to decreased activity, for example, (4-2)–(4-4) (*Table 2.4*). However, the use of side-chain protected Lys derivatives (e.g. the NH_2 terminus protected with benzyloxycarbonyl or picolinyl) led to increased reactivity to elastase with the optimal position for substitution being P_4, see (4-5)–(4-8).

More recent studies on a series of peptide, thioester substrates which have a P_2-Ala residue (not illustrated) have shown that HLE prefers by ~3-fold a P_1-Nva to P_1-Val residue [100]. Extension of the substrate studies to the S′-subsites of elastase was accomplished with a series of compounds which had at P_1 to P_4 a set of residues (including: Ala, Ser or Thr at P_1'; Ala, Pro or Ser at P_2'; and Pro at P_3' or P_4') designed to direct enzymatic cleavage to the P_1–P_1' bond. Using this region as a foundation, the substrates were sequentially extended into the P′ region. These studies revealed that the

Table 2.4. KINETIC CONSTANTS FOR HYDROLYSIS OF SYNTHETIC 4-NITROANILIDES BY HLE[a]

Compound	*Substrate*					k_{cat}/K_m[b] $(M^{-1}\ s^{-1})$
	P_5	P_4	P_3	P_2	P_1	
(4-1)	MeO-Suc	Ala	Ala	Pro	Val-NA	120,000
(4-2)	MeO-Suc	Lys	Ala	Pro	Val-NA	17,000
(4-3)	MeO-Suc	Ala	Lys	Pro	Val-NA	32,000
(4-4)	MeO-Suc	Ala	Ala	Lys	Val-NA	NR
(4-5)	MeO-Suc	Z | Lys	Ala	Pro	Val-NA	710,000
(4-6)	MeO-Suc	Ala	Z | Lys	Pro	Val-NA	260,000
(4-7)	MeO-Suc	Ala	Ala	Z | Lys	Val-NA	NR
(4-8)	MeO-Suc	Pic | Lys	Ala	Pro	Val-NA	790,000

[a]See footnote 'e' in *Table 2.1*.
[b]All data from [36].

S_1'-subsite of HLE prefers a P_1'-Ala or -Thr residue over -Ser and that recognition extended to include a P_3'-Pro residue [37].

PEPTIDE INHIBITORS

Monohalomethyl ketones

Peptidic monohalomethyl ketones inactivate serine proteinases by irreversibly reacting with the active-site histidine [101]. However, because of the reactivity pattern and the mechanism of action shown by these compounds, they are described in this section. Although initially prepared as inhibitors of other proteinases, monohalomethyl ketones contributed to the studies on elastase–ligand interactions. They supplied key data points on: (a) the effect of the peptide substrate length on the binding, and (b) the subsite specificity of PPE and HLE. Kinetic evaluation of these compounds indicated a two-step mechanism for enzymatic inhibition (*Equation 2.2*).

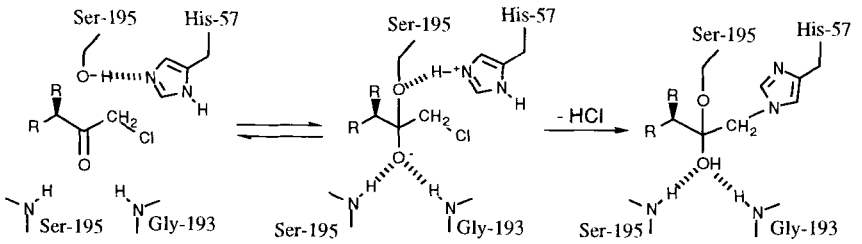


Figure 2.3. Mechanism of enzyme inactivation by peptide chloromethyl ketones.

Equation 2.2

$$E + I \underset{}{\overset{K_i}{\rightleftharpoons}} E{\cdot}I \xrightarrow{k_2} E \sim I$$

Initially, a reversible complex (E·I) forms, which then undergoes irreversible alkylation (E ~ I) [102]. Although both K_i and k_2 may be measured, this is done only with some difficulty, since eventually all of the inhibitor becomes irreversibly bound to the enzyme. Because of this, the more common way of assessing the potency of such inhibitors is by evaluating the ratio of k_{obs}/[Il. Under appropriate conditions this value remains constant over a range of inhibitor concentrations and is approximately equal to k_2/K_i. The detailed mechanism of inactivation is depicted in *Figure 2.3* and was first proposed by Kezdy with respect to chymotrypsin [103].

Circumstantial support for this mechanism was supplied by the fact that *N*-tosyl-Phe-CMK, a specific inhibitor of chymotrypsin, did not react with anhydrochymotrypsin [104]. Although both X-ray crystallographic and NMR studies supported the alkylated hemiketal as the structure of the inhibited enzyme, those studies did not prove whether alkylation or hemiketal formation occurred first [105, 98]. Carbon-13 NMR studies were also used to determine the pKa (7.88–8.1) of the hemiketal hydroxyl and this finding provided the first evidence that serine proteinases could stabilize the ionized form of the alkylated hemiketal, via hydrogen bonds in the 'oxyanion hole' [106, 107]. A series of more recent papers has confirmed that hemiketal formation precedes the alkylation step and has shown that the initial, reversible part of the interaction is made up of two discrete stages: (a) formation of a Michaelis complex, followed by (b) hemiketal formation [102, 108]. The requirement of an intermediate hemiketal may mean that chloromethyl ketone (CMK) inhibitors should be considered as 'transition-state' [109] analogue inhibitors (see discussion in section on Aldehydes).

The *in vitro* results found for CMK inhibitors of HLE (*Table 2.5*) mirror

Table 2.5. CHLOROMETHYL KETONE INHIBITORS OF HLE[a]

Compound	*Inhibitor*					$k_{obs}/[I]$ $(M^{-1} s^{-1})^{b}$	*Ref.*
	P_5	P_4	P_3	P_2	P_1		
(5-1)		Ac	Ala	Ala	Ala-CH_2Cl	0.3	110
(5-2)	Ac	Ala	Ala	Ala	Ala-CH_2Cl	1.0	110
(5-3)	Ac	Ala	Ala	Pro	Ala-CH_2Cl	4.4	110
(5-4)	Ac	Ala	Ala	Pro	Val-CH_2Cl	160.0	112
(5-5)	Suc	Ala	Ala	Pro	Val-CH_2Cl	320.0	112
(5-6)	MeO-Suc	Ala	Ala	Pro	Val-CH_2Cl	922.0	112

[a]See footnote 'e' in *Table 2.1.*
[b]k_{obs} value for runs at pH 6.5.

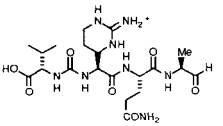
the findings reported for substrates. Extending from a tripeptide to a tetrapeptide, (5-1) versus (5-2), results in an increase in the rate of inactivation as does incorporation of a P_2-Pro, (5-2) versus (5-3) [110, 111]. Further increases in potency are achieved by replacing of the P_1-Ala residue with Val, (5-3) versus (5-4), and the P_5-Ac with either Suc or MeO-Suc, (5-5) or (5-6) respectively [112].

Several CMK inactivators have been examined *in vivo*. The relatively weak CMK (5-3) was tested in an elastase-induced emphysema model in the hamster. It was effective when administered i.p. at a total dose of 19 mg/hamster both *before and after* elastase exposure [113]. CMK (5-5) was effective i.t. at a dose of 0.5 mg/hamster when administered either 1 h before or 1 h after HLE exposure, in an identical model [114]. The most effective compound (5-6) worked following p.o. administration in a similar model in mice [115], or after i.t. administration in the elastase-induced acute haemorrhage model in hamsters [116]. Although peptide CMKs were the first low-molecular-weight inhibitors shown to be effective *in vivo* against elastase, they have consistently demonstrated toxic side-effects which have precluded their development [117, 118].

Aldehydes

Leupeptin, (6-1) (*Table 2.6*), the first peptide aldehyde inhibitor of serine and cysteine proteinases was isolated in 1969 from several species of *Streptomyces* [119]. It was only weakly active against trypsin and was inactive against elastases, but its discovery spurred continued screening of

Table 2.6. PEPTIDE ALDEHYDE INHIBITORS OF HLE[a]

Compound	*Inhibitor*					*K_i (μM)*	*Ref.*
	P_5	*P_4*	*P_3*	*P_2*	*P_1*		
(6-1)		Ac	Leu	Leu	Arg-H	ND	119
(6-2)						65.0	121
(6-3)		Ac	Pro	Ala	Pro-H	0.8 (PPE)	122
(6-4)		Ac	Pro	Ala	Pro-NH_2	Substrate	122
(6-5)	Suc	$AdaSO_2$ | Lys	Val	Pro	Val-H	0.11	125
(6-6)	Suc	Cbz | Lys	Val	Pro	Val-H	0.035	124

[a]See footnote 'e' in *Table 2.1.*

microbial cultures, which led to the isolation of elastatinal (6-2) [120, 121]. This natural product was considered, at that time, to be a relatively good reversible inhibitor of elastases. The finding that it was several hundred-fold more potent an inhibitor of PPE ($K_i \approx 0.24\,\mu$M) than HLE (Ki $\approx$ 50–80 μM) helped the efforts to determine the primary substrate specificities for these two enzymes.

Simultaneous with this work, the first synthetic aldehyde inhibitors of elastases were independently conceived by Thompson [122]. He had predicted that aldehydes, such as (6-3), would be 'transition-state' inhibitors of serine proteinases (PPE). The finding that aldehyde (6-3) was approximately 1000-fold more potent as an inhibitor of PPE than the corresponding alcohol and that it had approximately 5000-fold higher affinity for PPE than the corresponding primary amide substrate (6-4) provided strong evidence in support of his argument. Also, as was described for α_1-PI, the importance of the Ser-195 hydroxyl to the activity of these aldehydes was shown in a study wherein the dissociation constant between (6-3) and anhydro-PPE increased ~5000-fold as compared to its potency as an inhibitor of PPE [123].

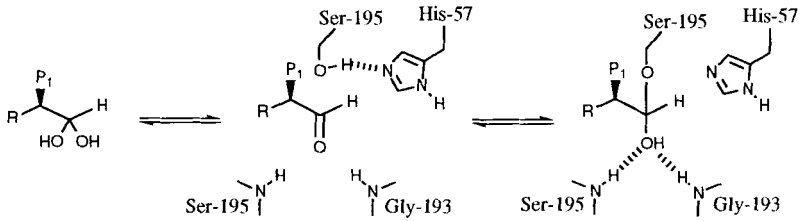


Figure 2.4. Mechanism of enzyme inhibition by aldehydes.

The rationale used to predict and/or explain the inhibition of serine proteinases by peptide aldehydes was, in part, based on the fact that aldehydes are relatively electrophilic. Therefore, addition of the Oγ of Ser-195 to an aldehyde carbonyl group should occur more readily than attack at a normal peptide (amide) linkage. Also, in contrast to ordinary amides, esters, or ketones, many aldehydes prefer to exist in aqueous solution as a tetrahedral addition product, for example, the hydrate in water. Confirmation that the peptide aldehydes were producing a hemiacetal 'transition-state analogue' (see *Figure 2.4*) via a covalent linkage to Ser-195 was obtained from several NMR studies using α-chymotrypsin and anhydro-PPE [126, 127]. Those studies also indicated that it is the free aldehyde, not the hydrate, which is the initially bound species and which is the target for nucleophilic attack by the Ser-195 hydroxyl [127].

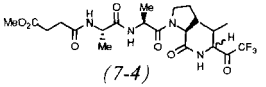
Optimization of the peptide backbone of these aldehydes to take advantage of the binding interactions in the S_1-S_4 subsites afforded potent inhibitors of HLE, for example, aldehyde (6-6) [124]. Concurrent with the increases in potency, the selectivity of these compounds also improved. For example, aldehyde (6-5) was inactive at 100 μM against other enzymes, including the serine proteinases trypsin, chymotrypsin, and cathepsin G [125]. Aldehyde (6-5) was compared with α_1-PI and was shown to be more potent *in vitro* (on a weight basis) and more stable towards oxidative inactivation by cigarette smoke [128].

Aldehyde (6-5) was also examined *in vivo* in both hamsters and mice [125]. Following i.t. administration to the hamster, it showed a lung retention half-life of 2 h. If administered via the i.t. route either 40 min. prior to or admixed with HLE it was effective at blocking both acute haemorrhage (AHA model) and development of emphysema (EMP model). Limited toxicological evaluation in mice revealed no overt effects following a single i.p. dose of 3.2 g/kg. Although interesting compounds were produced, aldehydes are subject to facile (chemical or metabolic) oxidation to the acid, and no examples have been reported to be in clinical development.

Trifluoromethyl ketones

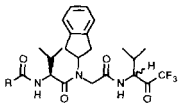
The peptide aldehyde series demonstrated the potential for carbonyl derivatives to be potent inhibitors of serine proteinases. The 200-fold increase in K_i values which was found in going from an aldehyde (7-1) (*Table 2.7*) to its corresponding methyl ketone (7-2) (*Table 2.7*) [129] implied that the physical properties of the carbonyl group were an important factor in determining the potency of the inhibitor. As a means of increasing both the stability and potency of peptide ketone derivatives Trainor and co-workers [130, 131] and Imperiali and Abeles [132] independently prepared the first peptide trifluoromethyl ketone (TFMK) inhibitors of serine proteinases in

Table 2.7. PEPTIDE TFMK INHIBITORS OF HLE[a]

Compound	 *(7-4)*					K_i *(μM)*[c]	*Ref.*
	P_5	P_4	P_3	P_2	P_1[b]		
(7-1)		Cbz	Val	Pro	Val-H	0.041	129
(7-2)		Cbz	Val	Pro	Val-Me	8.0	129
(7-3)		Cbz	Val	Pro	Val-CF_3	0.0016	129
(7-4)	MeO-Suc	Ala	Ala	Pro	Val-CF_3	0.014	133
(7-5)	Cbz	Ala	Ala	Pro	Val-CF_3	0.0016	133
(7-6)	Cbz	(Cbz) | Lys	Val	Pro	Val-CF_3	<0.0001	1f
(7-7)	Ada$(CH_2)_2$OC(O)		(OtBu) | Glu	Pro	Val-CF_3	0.00004	1f
(7-8)	*p*-$HO_2CC_6H_4C(O)$		Val	Pro	Val-CF_3	0.005	1f
(7-9)	*p*-$R^1C_6H_4C(O)$		Val	Pro	Val-CF_3[d]	0.0005	42
(7-10)	*p*-$R^2C_6H_4C(O)$		Val	Pro	Val-CF_3[d]	0.0005	42
(7-10)	*p*-$R^2C_6H_4C(O)$		Val	Pro	Val-CF_3[d]	0.038(IC_{50})	126

[a]See footnote 'e' in *Table 2.1*.
[b]The TFMKs are diastereomeric mixtures at the P_1-Val methine.
[c]Although these TFMKs are 'slow-binding inhibitors' of HLE these are the equilibrium K_i values.
[d]R^1 = *p*-$ClC_6H_4SO_2NHCO$–; R^2 = *p*-$BrC_6H_4SO_2NHCO$–.

Table 2.8. N-ALKYL GLYCINE PEPTIDE TFMK INHIBITORS OF HLE[a]

Compound		*$IC_{50}(\mu M)$*	*Ref.*
(8-1)	R = $PhCH_2O$–	0.365	136
(8-2)	R = *p*-(*p*-$ClC_6H_4SO_2NHCO)C_6H_4$–	0.084	136

[a]See footnote 'e' in *Table 2.1.*

general, and HLE in particular. This change was designed to increase the relative electrophilicity of the ketone and its preference for existing as a tetrahedral adduct. Due to the synthetic routes employed, all of the peptide-TFMK derivatives listed in *Tables 2.7* and *2.8* were mixtures of diastereomers, which are epimeric at the P_1-α-carbon. Not surprisingly, several studies have shown that activity resides in a single diastereomer [133, 134] which has the (*S*)-configuration at the P_1-α-carbon [135].

The electron deficient trifluoromethyl substituent increases the ketone's electrophilicity and, therefore, its susceptibility to nucleophilic attack by the Oγ of Ser-195. Although other carbonyl substituents, for example, CN or Cl groups, would also be electron withdrawing, the trifluoromethyl group is a relatively poor leaving group. Therefore, the TFMKs, unlike acyl chlorides or cyanides, will not progress to an acyl-enzyme complex. Application of the structure-activity relationships found in the peptide aldehydes and further optimization in the TFMK series led to potent and selective inhibitors. *In vitro*, the most potent compounds (7-5, -6 and -7) had very lipophilic *N*-protecting groups at P_4, or P_5 [1f, 130, 133].

Further exploration of the effect of variation of the peptide backbone on potency and specificity of the peptide-TFMK derivatives revealed that it was possible to retain activity if the P_2-Pro was replaced with an *N*-alkyl glycine residue, for example, (8-2) versus (7-10) [137, 136]. A similar replacement, of a C-terminal proline residue, had previously been shown to be effective in a series of potent angiotensin-converting enzyme (ACE) inhibitors [138]. In the anti-elastase series, molecular modeling studies showed that the extended binding region of HLE could accommodate the bulky *N*-alkyl group and that the inhibitor peptide chain may also adopt the conformation needed for good binding interactions with HLE [137].

The interaction between serine proteinases and TFMK derivatives has been the object of kinetic [139], NMR [133] and X-ray crystallographic studies [140]. Those studies revealed that the TFMK mechanism of action

was similar to that previously described for aldehydes (see *Figure 2.4*) and that such compounds are true 'transition-state analogues'. One difference was that some TFMKs, although very potent, were 'slow-binding inhibitors', for example $k_{on} = 9 \times 10^4\ M^{-1}s^{-1}$ for (7-9) [139, 141]. While the reason for this remains unproven, one possible cause [139] is found in the fact that the TFMKs are almost exclusively hydrated in aqueous solution, that is, they exist as the dihydroxyketal. They must dehydrate to the ketone form before the Oγ of Ser-195 can add to form the covalent adduct.

Since the equilibrium concentration of the TFMK ketone form is quite low, the stage also appeared to be set for the TFMKs to be weaker than expected based on their greater electrophilicity relative to the aldehydes. However, the pKa of the hemiketal hydroxyl group in a TFMK-chymotrypsin complex was shown to be ~4.0 [142]. This finding might explain the TFMK's greater potency since at physiological pH the hemiketal hydroxyl in the TFMK complexes, unlike that with the aldehydes, should exist as the deprotonated oxyanion. The presence of an ionized species (the hemiketal alkoxide) implies the existence of especially strong H-bonds between it and the NH groups of the amino acid residues in the oxyanion hole [143]. The estimated strength of such bonds is such that they would more than compensate for the decreased concentration of the 'active' inhibitor.

Although lipophilic P_4-substituents in the peptidyl-TFMKs afforded the most potent inhibitors, *in vitro*, these compounds were not optimal for use *in vivo*. This finding was not surprising since previous work had shown that large, polar compounds are retained in the lung better than small, nonpolar substances [144]. In addition to being very lipophilic, compounds such as (7-5, -6, -7) suffered from poor aqueous solubility. The solubility problem could be improved by the incorporation of carboxy-substituted groups at the N-terminal, for example (7-8). However this polar group led to apparent increases in K_i, as compared to the parent CBZ-protected analogue (7-5). The use of a bis-aryl acylsulphonamide-derived substituent afforded a balance between increased aqueous solubility and lipophilicity and also led to a decrease in K_i, for example (7-9 or 7-10) vs.(7-5) [26].

The acylsulphonamide group proved excellent for achieving prolonged duration of action *in vivo* after i.t. administration [42]. Pretreatment of hamsters with either i.t. (7-9) (ICI 200,880) or (7-10) (ICI 200,355), at doses of 21–420 μg/animal, produced dose- and time-dependent inhibition of HLE-induced effects, in the ALI model. Similarily, subcutaneously (s.c.) administered (7-9) afforded protection both in this model and in the EMP model [42]. The excellent overall profile of (7-9) resulted in its progression to clinical development [145]. More recently it was shown that the beneficial

effect of the acylsulphonamide substituent on the *in vivo* profile of peptide-TFMK HLE inhibitors could be extended to the *N*-alkyl glycine series (8-2)[136]. However, there have been no reports of the *N*-alkyl series progressing to clinical trials.

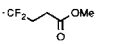
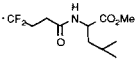
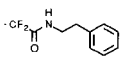
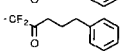
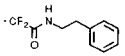
P′-Extended ketones

Although peptide TFMKs showed enhanced potency and chemical stability as compared to the peptide aldehydes, they are similarly structurally limited in that they cannot be extended in the P′ direction. Such a variation would be potentially desirable since several studies have indicated that HLE's binding region extends in the S' direction [37]. Structural modifications in this region might also result in improved physical and pharmacokinetic properties, for example, solubility and *in vivo* duration of action. The TFMKs could serve as a platform for appending P′ residues if one of the fluorine atoms was replaced with an extended chain, or if the entire trifluoromethyl group was replaced with an alternative electron-deficient group, for example, substituted acyl. A variety of such substitutions have been explored, giving rise to the following series of alternative electrophiles: α,α-difluoroketones (DFKs) [146]; α,α-difluoro-β-ketoamides (DFKAs), α,α-difluoro-β-diketones or α,α-difluoro-β-ketoesters [147, 133]; α-ketoamides [147]; α-ketoesters [148, 149]; α-diketones [147, 149]; and α-ketoheterocycles [129].

If the difluoroketone inhibitors (*Table 2.9*) are to be as effective as the TFMKs, then the binding interactions gained at the S′ subsites must compensate for the increase in K_i that occurs upon replacement of one of the TFMK fluorine atoms with a non-electronegative group, for example (9-1) vs. (9-2). As discussed in the TFMK section, this increase in K_i is expected due to the detrimental effect that a less electron-withdrawing substituent will have, on the formation and stability of the Ser-195 hemiketal adduct. Although the extended chain ketone (9-3) showed only a 3-fold decrease in K_i, as compared to ketone (9-2), extension to a Leu-derivative (9-4) afforded a 30-fold decrease. The net result, however, was that (9-4) was only equipotent to the parent TFMK (9-1).

If the replacement for the fluorine atom was also strongly electronegative, then it might be possible to retain the original binding affinity, derived from the stability of the hemiketal adduct, while also picking up additional binding interactions in the extended chain region. The overall result might then be even more potent inhibitors. This possibility was proven with DFKAs and α,α-difluoro-β-ketones, for example, compare TFMK (9-5) to compounds (9-9) and (9-10). It was also demonstrated that excellent

Table 2.9. P′ EXTENDED PEPTIDE KETONE INHIBITORS OF HLE[a]

Compound	*Inhibitor*					*K_i (μM)*	*Ref.*
	P_5	*P_4*	*P_3*	*P_2*	*P_1*[b]		
(9-1)	Cbz	Ala	Ala	Pro	Ala-CF_3	0.7	146
(9-2)	Cbz	Ala	Ala	Pro	Ala-CF_2H	20.0	146
(9-3)	Cbz	Ala	Ala	Pro	Ala 	7.0	146
(9-4)	Cbz	Ala	Val	Pro	Val 	0.7	146
(9-5)		Cbz	Val	Pro	Val-CF_3	0.0019	147
(9-6)		Cbz	Val	Pro	Val-$CONH_2$	0.0018	147
(9-7)		Cbz	Val	Pro	Val-COBu	0.0016	147
(9-8)		Cbz	Val	Pro	Val-CO_2Et	0.0006	147
(9-9)		Cbz	Val	Pro	Val 	0.0004	147
(9-10)		Cbz	Val	Pro	Val 	0.00023	147
(9-11)	*p*-$R^1C_6H_4C(O)$[c]		Val	Pro	Val 	0.00004	147
(9-12)		Cbz	Val	Pro	Val 	0.0006	129

[a]See footnote 'e' in *Table 2.1.*
[b]The TFMKs are diastereomeric mixtures at the P_1 methine whereas the other derivatives are single (*S*)-enantiomers at P_1.
[c]R^1 = $C_6H_5SO_2NHCO$–.

potency could be achieved if the trifluoromethyl group was replaced with an acyl derivative, e.g. compare TFMK (9-5) with α-ketoamide (9-6), α-diketone (9-7) and α-ketoester (9-8).

These extended-chain ketones are believed to interact with HLE similarily to the aldehyde and TFMK series of inhibitors. Although studies showing that they are true 'transition-state analogue' inhibitors have not been reported, the formation of the covalent hemiketal adduct was documented in an X-ray crystallographic study of a DFK bound to PPE [150]. One notable finding is that the acidic N-terminal substituents first used by Trainor and co-workers to produce TFMKs with good *in vivo* profiles

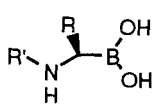
(following topical administration) also afforded good *in vivo* activity in the DFKA series [147]. For example, i.t.-administered (9-11), at 30 μg/hamster, showed inhibition of HLE in the ALI model for prechallenge intervals of up to 24 h.

Boronic acids

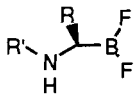
Boronic acids (5a) were among the first examples of low-molecular-weight, reversible inhibitors of serine proteinases [151, 152]. Significant inhibition was initially demonstrated against α-chymotrypsin. Unlike the carbonyl-derived reversible inhibitors, which require a polypeptide or peptide-like chain, activity was found with simple alkylboronic acids (e.g. the K_i value for $PhCH_2CH_2B(OH)_2$ with α-chymotrypsin was $\approx$ 40 μM) [153]. Weak inhibition of elastase (PPE) was first reported for a series of arylboronic acids, for example, (10-1) [123]. Some of the boron-based inhibitors (*Figure 2.5*) were tested as either the difluoroboranes (5b) or as the pinacol boronate esters (5c). These modifications were employed because they were more readily synthesized and/or purified than the boronic acids. For both of these derivatives inhibition was shown to be due to in situ hydrolysis to the parent boronic acid (5a) [154, 155].

As in the peptide substrate or electrophilic carbonyl-based inhibitor series, the addition of a peptide chain to take advantage of binding interactions in the S_1–S_5 subsites of elastase results in increased potency and selectivity, for example, (10-1) vs. (10-2) (*Table 2.10*) [154]. In contrast to the above two series, boronic acid (10-5) whose P_1-Phe residue was chosen for it to be a specific α-chymotrypsin inhibitor (final K_i = 0.16 nM vs. chymotrypsin), retained good activity against both HLE and PPE. Also each of the peptide boronic acids corresponding to the better substrates for α-chymotrypsin, PPE, and HLE (for example, (10-6, -7, and -8), respectively) were slow-binding inhibitors of those enzymes [155].

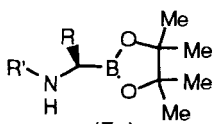
At first it was believed that enzyme inhibition by boronic acid derivatives was due to reversible covalent bond formation between boron and His-57 [151, 156]. Then several studies showed that with some inhibitors,



(5a)



(5b)



(5c)

Figure 2.5. Boron derived inhibitors: (5a) boronic acid; (5b) difluoroborane; (5c) pinacol boronate ester.

Table 2.10. BORONIC INHIBITORS OF HLE AND PPE[a]

Compound	*Inhibitor see Figure 2.5 R′ = P_5–P_2–*					*HLE* K_i *(μM)*	*PPE* K_i *(μM)*	*Ref.*
	P_5	P_4	P_3	P_2	P_1			
(10-1)			meta-NO_2PhB$(OH)_2$			ND	100.0	123
(10-2)			Cbz	Ala	boro-Val-OH	ND	1.1	154
(10-3)			Cbz	Ala	boro-Ile-OH	ND	0.12	154
(10-4)			Cbz	Ala	boro-Ile-F	ND	0.10	154
(10-5)	MeO-Suc	Ala	Ala	Pro	boro-Phe-OH	0.35	0.27	155
(10-6)	MeO-Suc	Ala	Ala	Pro	boro-Ala-OH	0.079	0.0182[b] 0.00032	155
(10-7)	MeO-Suc	Ala	Ala	Pro	boro-Val-OH	0.015[b] 0.00057	0.0030[b] 0.00025	155
(10-8)	MeO-Suc	Ala	Ala	Pro	D,L-boro-Val-pinacol ester	-0.060[b] 0.00057	ND	155

[a]See footnote 'e' in *Table 2.1*.
[b]Indicates that slow-binding inhibition was observed and that both the initial (larger number) and final K_i values are reported.

complexes are formed in which the boron atom is covalently attached to Ser-195 [157–159, 123]. Formation of either of these complexes is possible because trigonal boron compounds contain a vacant 2p electron orbital, which readily reacts with nucleophiles to form a tetrahedral adduct. Irrespective of the original rationale behind the inhibitor design, the boron-containing inhibitors show affinity for serine proteases which is up to 4 orders of magnitude higher than that of the corresponding substrates [160, 155]. Recently, Bachovchin and co-workers developed a unifying hypothesis which reconciles the recent and earlier observations (see *Figure 2.6*) [161, 162]. Very potent boronic acid inhibitors form a tetrahedral adduct with the active-site Ser-195 (type-1 inhibitor). 'Poor' inhibitors also covalently bond to Oγ Ser-195; however, in this case they eliminate a molecule of water and form a trigonal, planar adduct (type-2 inhibitor).

The type-1 complex is a 'transition-state' mimic and such boronic acid inhibitors are potent inhibitors. The greater affinity of these boronic acids as compared to the corresponding peptide-TFMKs (for example, compare (10-7) with TFMK (7-4) which had a K_i = 14 nM for HLE) might be explainable by the fact that in the boronic acid–enzyme complex there is an

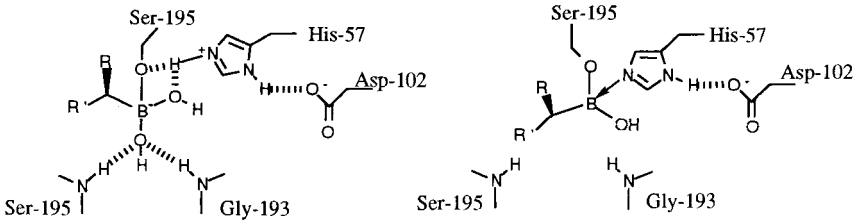


Figure 2.6. Structure of boronic acid–enzyme complexes [240].

additional hydrogen bond between His-57 and one of the boronate oxygens. In the type-2 complex the N_ε of His-57 is in an axial position 2.2 Å from the boron and it appears to form a dative bond with the boron. The reason such inhibitors fail to react in a substrate-like manner to afford a transition-state mimic is that they do not properly fit the S_1 specificity pocket.

Several *in vivo* studies have been reported for compound (10-8). In the hamster EMP model the effect of PPE was significantly attenuated by (10-8), administered at 200 mg/kg body weight, either i.p. 15 min. prior to, or admixed with, elastase [163]. In addition to this pharmacological model, the enzyme inhibitory capacity (EIC) of hamster plasma was determined after either i.p., p.o., intramuscular, or subcutaneous dosing of (10-8). The EIC values provided a measure of the plasma concentration of (10-8). Following p.o. administration, the change in plasma EIC was negligible, but following the three other routes of administration a 10-fold increase in the plasma EIC occurred. These studies also showed that the half-life of (10-8) was ~2h, regardless of the mode of administration. A second set of studies in which (10-8) was given at different times and amounts demonstrated that the degree of effectiveness against PPE was both dose and time dependent [164]. In surprising contrast to the effectiveness of (10-8) against PPE, similar studies using HLE showed no protection and indicated that the HLE insult was exacerbated by (10-8) [165]. Limited toxicity studies in mice, using (10-8) given intravenously, showed no mortality, behavioural changes, or other symptoms of acute toxicity [166]. Although progression of (10-8) to clinical studies has not been reported, the potential clinical utility of boronic acids is indicated by the fact that DuP714, a related boronic acid inhibitor of thrombin from the same labs, has progressed to the clinic [167].

ACYCLIC NONPEPTIDE ACYLATING AGENTS

The activity of most irreversible inhibitors and inactivators of serine

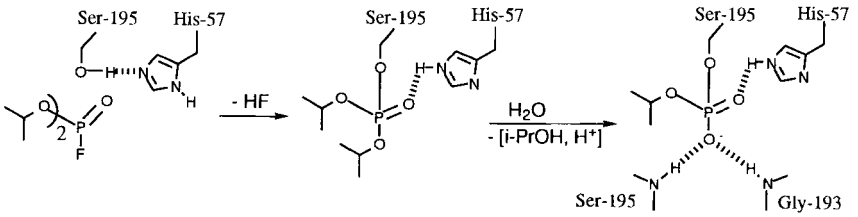


Figure 2.7. Mechanism of enzyme inactivation by phosphofluoridates.

proteinases is based on their ability to react with the catalytic triad. The nonpeptidic inhibitors and inactivators are unable to make full use of the potential interactions in the extended binding pocket of the enzyme, so they must form a covalent bond with one of the active-site residues to achieve significant potency.

The first nonpeptide, irreversible inactivators of serine proteinases were phosphoryl fluoride derivatives such as diisopropylphosphofluoridate (see *Figure 2.7*) [168]. It was shown that the phosphofluoridates stoichiometrically inactivate serine proteinases, by coupling with the active-site serine hydroxyl group. They have since been used as a diagnostic tool for the classification of serine proteinases. Similarily, both aryl and aralkyl sulphonyl fluorides were determined to be inactivators, forming a sulphonyl enzyme derivative at Ser-195 which is resistant to hydrolysis.

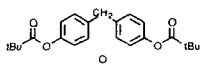
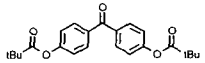
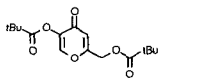
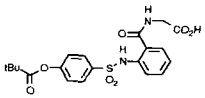
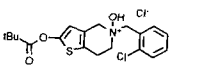
For such inactivators to be effective both *in vitro* and *in vivo*, there are several conditions which must be met. First, they must be sufficiently stable that they are not spontaneously hydrolyzed or cleaved by esterases. Second, the acyl-enzymes which they form must be relatively stable to deacylation. Third, they should have good enzyme selectivity. Both the phosphofluoridates and sulphonyl fluorides are too hydrolytically reactive and nonselective to be examined in *in vivo* models. Recently, several (α-aminoalkyl)phosphonate diphenyl esters were explored [169] in order to address some of these issues. However, as no *in vivo* results were reported with them, neither they, nor the phosphonyl fluorides nor the sulphonyl fluorides will be discussed further in this review.

Acylating agents (for example, acyl chlorides or activated esters) have the potential to inhibit serine proteinases similarly to the sulphonyl and phosphorylating agents by binding to and blocking Oγ of Ser-195. A group at Searle used the random-screening discovery that some unstable steroidal enol pivalates were potent, selective, competitive inhibitors of HLE as the starting point for development of a series of inhibitors which were pivalic acid esters of phenol and kojic acid [170, 171]. These compounds, (for

example, 11-1, -2 and -3) (*Table 2.11*) are potent inhibitors that are selective for HLE over PPE and are more stable to hydrolysis than the starting steroidal enol pivalates. A group at Ono Pharmaceuticals has reported on a related *p*-substituted phenylpivalate, (11-4) (ONO-5046), which is ~100-fold more active against HLE than PPE [172]. The difficulty of comparing the potency of compounds reported by different groups is clearly illustrated by comparison of (11-2) and (11-4). The K_i values reported for these two compounds imply that (11-2) is ~3-fold *more* potent an inhibitor than (11-4) whereas the IC_{50} values imply that it is ~3-fold *less* potent. Simultaneously with this work, a team at Sanofi reported the discovery of (11-5) (SR 26831) which had very similar *in vitro* characteristics to (11-4) [173].

All of these compounds are believed to work via pivaloylation of the active-site Ser hydroxyl group. Since the inactivated enzyme should in all cases be identical, the acyl-enzyme should show the same resistance to reactivation no matter which inhibitor is utilized. However, these reports implied that this was not the case since similar studies in which enzyme, inhibitor and substrate were mixed and allowed to sit for extended periods of time revealed that active enzyme was regenerated with either (11-2) or (11-3) [170] but not with (11-5) [173]. Detailed comparison of these reports revealed that this discrepancy might have come about since the concentration of the inhibitor in the studies using (11-5) was higher than in the studies

Table 2.11. PIVALOYL ACYLATING AGENTS

Compound	*Structure*	*K_i (μM) HLE*	*IC_{50} (μM) HLE*	*IC_{50} (μM) PPE*	*Ref.*
(11-1)		ND	0.46	ND	170
(11-2)		0.071	0.12	inactive at 10.0	170
(11-3)		0.09	0.27	inactive at 10.0	170
(11-4) ONO-5046		0.2	0.044	5.6	172
(11-5) SR-26831		0.36	0.080	5.0	173

using (11-2) or (11-3), and neither study reported on the susceptibility of the inhibitors to spontaneous hydrolysis in the aqueous buffer.

Unlike the Searle series, pivalates (11-4) and (11-5) had good aqueous solubility which might explain why *in vivo* results have only been reported for the last two compounds. In the hamster AHA model, i.t. (11-4), administered 5 min. prior to HLE insult, significantly suppressed lung haemorrhage with an ID_{50} of 82 μg/kg. In a new model in guinea pigs, i.v. (11-4), administered 30 s prior to intradermal HLE specifically inhibited elastase-induced increases in skin capillary permeability over the course of a 30 min. study period [172]. In a rat version of the AHA model, i.t. (11-5) administered 15 min. prior to HLE insult totally suppressed lung haemorrhage at a dose of 1 mg/kg (no i.t. dose response data was reported). In the same model both i.v. and p.o. (11-5) were shown to significantly suppress lung haemorrhage at doses greater than 0.25 mg/kg (15 min. prior to HLE) and 30 mg/kg (1 h prior to HLE), respectively. However, in both cases the maximal degree of protection that could be obtained was ~50%. Following a single i.v. dose of 1 mg/kg, (11-5) inhibited subsequent HLE induced haemorrhage for prechallenge intervals extending up to 90 min.

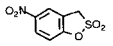
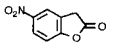
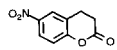
HETEROCYCLIC INHIBITORS/INACTIVATORS

Lactone and coumarin based inhibitors

The basis for exploring heterocycles as inhibitors of HLE came from the discovery that some heterocycles, for example, sultones (12-1, *Table 2.12*) [174], oxazolinones (12-2) [175], or lactones (12-3) and (12-4) [176], were alternate substrates for serine proteinases. The proteinases reacted with these compounds to: (a) cleave the heterocyclic ring, (b) form an intermediate acyl-enzyme and (c) then hydrolyze the acyl-enzyme by the normal catalytic mechanism to release active enzyme and the ring-opened heterocycle.

For these compounds to act as alternate-substrate inhibitors, the deacylation steps (the total k_{off}) of the catalytic process must be slowed down as compared to the acylation step (k_{on}). The fact that *o*-toluene-sulphonyl fluorides form a similar aryl sulphonate with Ser-195 as do the sultones, but are irreversible acylating/inactivating agents instead of being substrates, implied that slight changes in the structures of other heterocyclic substrates might be able to convert them to inhibitors. The kinetic processes for both alternate-substrate inhibitors ($k_d > k_2 = 0$) and dual acting inhibitors ($k_d \geq 0 < k_2$) are summarized in *Equation 3*:

Table 2.12. HETEROCYCLIC SUBSTRATES FOR α-CHYMOTRYPSIN

Compound	*Structure*	k_{cat}/K_m $M^{-1}\ s^{-1}$	*Ref.*
(12-1)		~31,000	174a
(12-2)		~66,000	175
(12-3)		9,000	176
(12-4)		7,800	176

Equation 3:

$$E + I \rightleftharpoons E \cdot I \rightleftharpoons E \sim I \begin{cases} \xrightarrow{k_d} E + I^* \\ \xrightarrow{k_2} \underline{E \sim I} \end{cases}$$

For alternate-substrate inhibitors, the enzyme (E) and inhibitor (I) first interact to give a reversible Michaelis complex (E • I). This complex rapidly progresses to a relatively stable acyl-enzyme (E ~ I) which may slowly either revert back to the active enzyme (E) and the intact inhibitor (I) or continue on via the normal catalytic machinery to the active enzyme (E) and the modified inhibitor (I*). With dual-acting inhibitors the acyl-enzyme contains a second reactive functionality which acylates or alkylates a second amino acid residue in the enzyme active-site, while the compound is still tethered to Ser-195 [177], resulting in inactivated enzyme ($\underline{E \sim I}$).

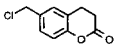
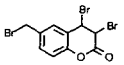
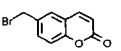
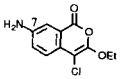
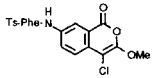
A more narrowly defined group within this second set of compounds is the set of mechanism-activated inhibitors. These are compounds which do not have a second pre-existing reactive functionality. Rather, they use the normal catalytic machinery of the enzyme to generate, or unmask, a reactive species in the acyl-enzyme intermediate (E ~ I). This new species then alkylates a second, suitably placed, active-site residue and permanently inactivates (binds to) the enzyme (even if deacylation of Ser-195 subsequently occurs). Efficient mechanism-activated inhibitors are those which have a high ratio of alkylation (k_2) to release (k_d) of the active enzyme. Because the second reactive functionality is only generated in the active site,

these compounds may show a relatively high degree of selectivity. If the acyl-enzyme cleaves before the alkylation step occurs, the original compound is considered a paracatalytic or pseudo-activated inactivator. This distinction may be demonstrated by preparation of the appropriate ring opened analogue and comparison of its activity with that of the parent heterocycle.

Incorporation of a benzylic halide into the structure of the alternate-substrate lactone (12-4) led to the bifunctional lactones (13-1, *Table 2.13*), and (13-2), which showed rapid and irreversible inactivation of α-chymotrypsin and PPE [178]. It was postulated that the intermediate acyl-enzyme formed from attack of Ser-195 on the lactone carbonyl dehydrohalogenated to form a reactive quinone methide that coupled with His-57. If this mechanism were followed, then lactone (13-2) would be an example of a mechanism-activated inhibitor. However, lactone (13-2) is sufficiently reactive as an alkylating agent to directly couple with imidazole while the lactone ring is intact. Because of this, it is not clear, from the published data, whether acylation of Ser-195 precedes alkylation, a prerequisite for this compound to be confirmed as a mechanism-activated inhibitor. Interestingly, the corresponding coumarin (13-3) was both less potent and only provided partial inactivation of α-chymotrypsin [179, 180]. It was shown that the lactone linkage in this coumarin was stable in the presence of α-chymotrypsin and that the modified enzyme retained its intact active-site. These facts led to the postulate that, like the action of phenacyl bromides or benzyl bromides on α-chymotrypsin, the partial inactivation by (13-3) involves alkylation of Met-192 [179].

Powers followed these early reports of lactone-based inhibitors with a detailed exploration of isocoumarin-based inhibitors, discovering that introduction of a chlorine atom at the 3-position, for example, compound (13-4), resulted in good inhibition of HLE [181]. This was somewhat surprising since isocoumarin itself shows no inhibition of HLE, PPE or chymotrypsin, nor is it a substrate of chymotrypsin [184]. The rationale used to explain this difference was that the introduction of an electron-withdrawing chlorine substituent makes the isocoumarin lactone more susceptible to nucleophilic attack and cleavage. In support of this view, introduction of a second chlorine atom at the 4-position led to faster inactivation rates [184]. Even better results were obtained with 3-alkoxy-4-chloroisocoumarins, for example, (13-5) [182]. Addition of a 7-amino substituent resulted in isocoumarin (13-6) [185]. The $k_{obs}/[I]$ values obtained with this compound against both HLE and PPE are smaller than those for (13-5) but, unlike (13-4) or (13-5), it is an irreversible inhibitor. Substitution of the amino group with *N*-tosyl-Phe gave (13-7), which was both highly

Table 2.13. LACTONE AND COUMARIN BASED INHIBITORS OF SERINE PROTEINASES[a]

Compound	*Structure*	$k_{obs}[I]$ $(M^{-1} s^{-1})$			*Ref.*
		α-CHYMO	*HLE*	*PPE*	
(13-1)		'efficient'[b]	ND	'efficient'[b]	180b
(13-2)		3,000	ND	'efficient'[b]	179
(13-3)		12	ND	'no effect'[b]	179
(13-4)		163	3,900	512	181
(13-5)		610	43,000	1,440	182
(13-6)		270	9,420	700	182
(13-7)		150	190,000	6.480	182
(13-8)		700[c]	34,700[c]	ND	183
(13-9)		12,000[c]	37,500[c]	ND	183

[a]See footnote 'e' in *Table 2.1.*
[b]Efficient and no effect mean >95% and 0% inhibition respectively, at pH 7.0, 4-5 μM enzyme, 195 μM compound. However, the detailed kinetics were not determined, as reported [180].
[c]k_a/K_i which uses similar units (M^{-1} s^{-1}) to, but is different from, $k_{obs}/[I]$.

effective as an inhibitor of HLE and was quite selective, since it did not inhibit several other enzymes and inhibited chymotrypsin quite slowly [182]. Although spontaneous reactivation of HLE or PPE which had been inactivated by any of the 3-alkoxy-7-amino-chloroisocoumarins was not observed, treatment with hydroxylamine resulted in complete reactivation for enzymes inactivated with (13-7) but only ~40% reactivation for enzymes inactivated with (13-6) [185].

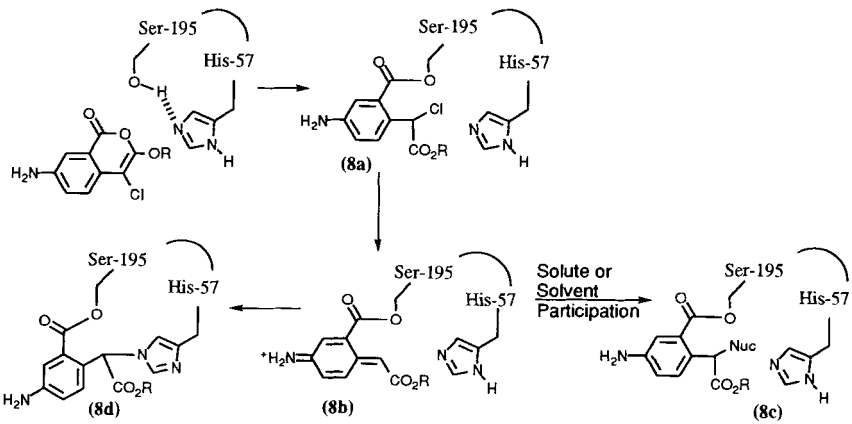


Figure 2.8. Mechanism of enzyme inactivation by 7-amino-4-chloroisocoumarins [240].

An explanation of these results and determination of the mechanism(s) of inhibition by the isocoumarins required a complex series of kinetic analyses and X-ray crystallographic studies [186]. These studies showed that the mechanistic pathway (see *Figure 2.8*) was pH-dependent [187] and that different forms of the inhibited enzymes, illustrated by (8a), (8c) and (8d), could be isolated. Ring-opening results in formation of an intermediate acyl-enzyme (8a), which, in some cases, can be isolated but which can also eliminate chloride to produce a reactive quinone imine methide (8b). This reactive intermediate is either trapped by solute or solvent, to produce a second acyl-enzyme (8c) [188] or alkylated by His-57 to produce an irreversibly inactivated enzyme (8d) [189]. The ratio between (8c) and (8d) has been shown to vary widely.

An alternate approach, which also uses enzyme-catalyzed ring-opening of a lactone to generate a mechanism-activated inhibitor, was developed by Katzenellenbogen and his co-workers [183], who found enol lactones, exemplified by (13-8) and (13-9), to be potent, selective inhibitors of HLE. The haloenol lactone (13-9) was an irreversible inactivator of HLE and chymotrypsin, and after exposure to (13-9), active enzyme could not be regenerated even upon treatment with hydrazine. Enol lactone (13-8), on the other hand, was an alternate-substrate inhibitor, which produced only transient inhibition of HLE and chymotrypsin. These results have been interpreted to mean that, with the halo-substituted compounds, ring opening results in formation of an acyl-enzyme that contains a reactive halomethyl ketone, which then alkylates His-57. That these compounds

were not pseudo-activated or paracatalytic inactivators was proven by preparation and testing of the ring-opened carboxy halomethylketones. It was also noted that the enzyme specificity of these compounds was established by the 3-substituent, with 3-aryl groups affording selectivity for α-chymotrypsin and a 3-isopropyl group being selective for HLE.

4H-3,1-Benzoxazin-4-ones

The desire to find a heterocyclic inhibitor that balanced hydrolytic stability, enzyme reactivity, and acyl-enzyme stability better than the above lactones and coumarins resulted in explorations of a variety of other oxycarbonyl-containing heterocycles including derivatives of isatoic anhydride [190], 6-chloropyrone [191], quinazoline [192], anthranilate [192], isobenzofurenone [193], and benzoxazinone.

The most comprehensive studies have been on 4*H*-3,1-benzoxazin-4-ones. Based on de Jersey's [175] report on the cleavage of oxazolinones, Alazard *et al.* [194] recognized that some benzoxazinones might be inhibitors of serine proteinases. He reported in 1973 that benzoxazinone (14-1, *Table 2.14*) was an alternate-substrate inhibitor, and that (14-2) was a mechanism-activated inactivator of α-chymotrypsin, which appeared to alkylate His-57. Interest in these compounds lapsed until 1982, at which time it was noted that benzoxazinones were inhibitors of PPE and HLE and that significant increases in potency were achievable by changing the 2-substituent, for example, (14-4) [192]. This report prompted a resurgence of interest in the benzoxazinones, with many of the more recent contributions on *in vitro* SAR and mechanism coming from a group at Syntex [195].

The mechanism of inhibition by benzoxazinones (*Figure 2.9*) is believed to be similar [196, 197] to that of the alternate-substrate isocoumarins, and formation of a covalent acyl-enzyme complex with PPE has been confirmed by X-ray crystallographic studies [198]. However, when R^2 is a hydrogen atom, deacylation of the acyl-enzyme via intramolecular ring closure can either reform the starting benzoxazinone (O attack) or lead to an isomeric quinazolinedione (N attack). It was shown for HLE and α-chymotrypsin that formation of the quinazolindione occurs faster than 'normal' hydrolysis to the anthranilic acid.

Just as theorized for the 3-chloroisocoumarins, the change in potency upon replacement of the 2-methyl group (14-1) by a 2-trifluoromethyl group (14-3) is believed to be due to increased electrophilicity of the ring carbonyl group. This change, however, also increases the hydrolytic instability of the parent ring system. The relative potencies of the series of homologous R^2-substituted benzoxazinones in *Table 2.14* are attributed to the stabiliza-

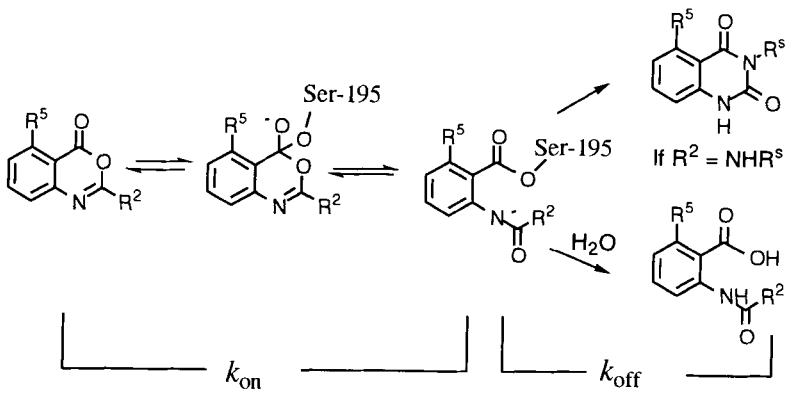


Figure 2.9. Mechanism of enzyme inhibition by benzoxazinones [240].

tion of the ring cleavage leaving group imparted by the substituent. Upon ring opening, isomerization of the leaving group to an *ortho*-amidobenzoate converts the activated acylating species to an acyl-enzyme which is deactivated towards deacylation.

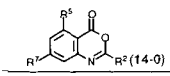
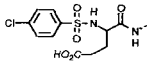
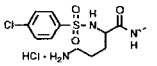
It was hypothesized that addition of a substituent at the 5-position would result in increased potency by decreasing k_{off} without significantly changing k_{on} [195]. The carbonyl group and the 5-substituent in a benzoxazinone are required to be coplanar, so a 5-substituent should not significantly hinder nucleophilic addition to the carbonyl group by Ser-195 and thus, should not effect k_{on}. After ring opening, the presence of two-flanking substituents in the acyl-enzyme product forces the arylcarbonyl group to rotate out of the plane of the aromatic ring. The acyl-enzymes formed from non-substituted benzoxazinones are only moderately hindered on one face and they readily deacylate. In the case of a 5-substituted benzoxazinone the acyl-enzyme is a 2,6-disubstituted benzoate in which the C = O group is orthogonal to the benzene ring and both of its faces are hindered towards nucleophilic attack, thus decreasing k_{off}. This strategem proved to be very effective at increasing potency, for example, (14-4) vs.(14-1) or (14-5) vs.(14-3). Detailed analysis of the enzyme inhibition kinetics revealed that ~2.7-fold of the increase in the pK_i of these compounds was due to an unexpected increase in k_{on}, while the remainder of the increase was due to the predicted decrease in k_{off} [199]. Later, ^{13}C NMR studies supplied evidence that the change in k_{on} was due to the relief of strain from a peri-interaction between the carbonyl oxygen and the C^5-substituent upon ring opening [200].

Although both 2-amino and 2-oxy substituents were predicted to increase

potency via increases in k_{on}, the 2-NH_2 analogue proved to be weaker than the parent 2-methylbenzoxazinone (14-6) vs. (14-1). This result was shown to be due to increases in k_{off} due to facile intramolecular deacylation with formation of a quinazolinedione. Hindering the amine with an isopropyl substituent resulted in a large increase in pK_i^* due almost exclusively to a decrease in k_{off} [201,202]. The 2-oxy-substituted series does not have this deacylation pathway available, and combination with a C^5-ethyl substituent led to the most potent example (14-10).

Recently, a group at Teijin Pharmaceuticals reported on two closely related compounds (14-11) (TEI-5624) and (14-12) (TEI-6344) [203]. Although these two compounds are significantly less active *in vitro* than the best compounds reported by the Syntex group, *in vivo* they showed promising activity. Measurement of their *in vivo* efficacy was limited to i.t., studies since after an i.v. dose both compounds were rapidly removed from the circulation ($t_{1/2} \approx 5$ min.) while after i.t. administration they had $t_{1/2}$ values of 85 and 240 min., respectively. In the hamster ALI model, both of

Table 2.14. BENZOXAZINONE INHIBITORS OF HLE

Compound				pK_i^{*a}	*Ref.*
	R^7	R^2	R^5		
(14-1)	H	Me	H	5.32	199
(14-2)		CH_2Br	H	6.02[b]	192
(14-3)	H	CF_3	H	6.77	199
(14-4)	H	Me	Et	6.92	199
(14-5)	H	CF_3	Me	7.54	199
(14-6)	H	NH_2	H	4.20	199
(14-7)	H	NH_2	Et	5.29	199
(14-8)	H	NH-iPR	Et	9.03	199
(14-9)	H	OEt	H	8.19	199
(14-10)	H	OEt	Et	10.37	199
(14-11)		NH-iPr	Me	~8.16	203
(14-12)		NH-iPr	Me	~7.79	203

[a]pK_i^* = the negative log of the K_i^* which has been measured in units of M^{-1}.
[b]Also reported as an inactivator of α-chymotrypsin with $k_2/K_i = 160\ M^{-1}s^{-1}$ [194].

these compounds significantly suppressed pulmonary haemorrhage when administered i.t. at 1 mg/kg, either 30 or 240 min. prior to challenge with i.t. HLE (1 mg/kg). The development of HLE-induced emphysema in hamsters (EMP model) was also prevented by i.t. (14-12) (1 mg/kg) given 7 h after i.t. HLE (1 mg/kg) administration.

Saccharin derivatives

Although the normal target of a serine proteinase is an amide linkage, most of the heterocyclic compounds which have been studied as HLE inhibitors have been lactone (ester) derivatives. In part, this may have been due to early studies by Kaiser [204], in which several lactams (amides) were examined as potential substrates/inhibitors of serine proteinases and were found not to significantly acylate the enzymes. The first detailed studies of lactam-based inhibitors of serine proteinases were on a series of *N*-acylsaccharins (15-0) [205]. It had been anticipated during the design of these compounds that they would be simple acyl-transfer agents, similar to the pivaloyl derivatives discussed above. However, it was demonstrated that the heterocyclic amide bond in these compounds is cleaved by elastase (see *Figure 2.10*) to produce a long lived acyl-enzyme (10a).

In the initial set of acylsaccharins it was shown that selectivity between chymotrypsin and PPE varied from ~2 / 1 to ~30 / 1 depending on the acyl group R^1, e.g. (15-1) vs. (15-3, *Table 2.15*). This was an interesting finding, because many of the lactone-based inhibitors had shown quite limited selectivity. Further work by the same researchers documented that the acyl substituent could be replaced by an aryl group (15-4), with retention of similar levels of *in vitro* activity [206]. More recently a group at Sterling has incorporated a leaving group into the saccharin N-substituent [207]. This

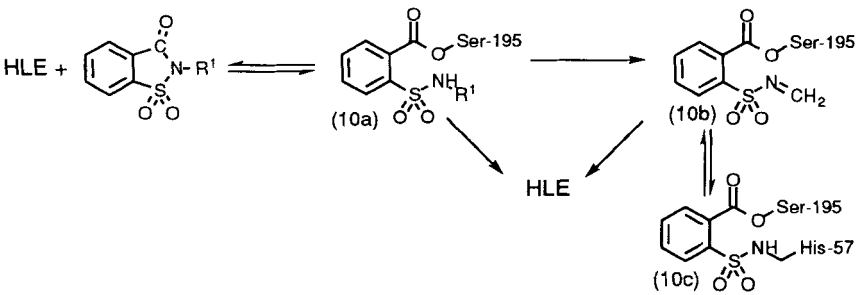


Figure 2.10. Mechanism of enzyme inhibition by saccharin based inhibitors. Only if R^1 contains a leaving group, eg. (15-4), does the inhibitor progress to a dicovalently linked enzyme.

Table 2.15. *SACCHARIN BASED INHIBITORS OF SERINE PROTEINASES*[a]

Compound		*15-0*	*IC_{50} or $\{K_i^*\}$ (μM)*			*Ref.*
	-R^2	*-R^1*	*α-CHYMO*	*HLE*	*PPE*	
(15-1)	-H		0.30	0.42	0.72	205
(15-2)	-H		0.07	0.36	0.58	205
(15-3)	-H		0.17	2.4	5.2	205
(15-4)	-H		0.5 {0.77}	1.0 {2.16}	ND ND	206 206
(15-5)	-H		ND	{0.015}	ND	207
(15-6)	-Et		{0.09}	{0.002}	{0.13}	207

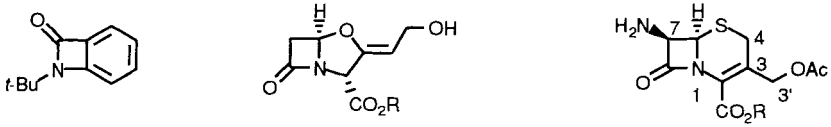
[a]See footnote 'e' in *Table 2.1.*

series of compounds, for example, (15-5), is reported to yield much more potent inhibitors. It was hypothesized that they are mechanism-activated inhibitors which form two covalent bonds to the enzyme [208]. Just as had been demonstrated for benzoxazinones, for example, (14-4) vs. (14-l), replacement of the peri-position hydrogen (R^2 = H) with an ethyl substituent yielded (15-6) which exhibited increased potency and selectivity opposite HLE.

Demonstration of *in vivo* activity by the saccharins was illustrated with (15-2) which was examined in the EMP model in hamster. It showed dose-related inhibition of PPE-induced emphysema when given i.t. (0.03–1.0 mg/ animal) 15 min. prior to PPE (0.1 mg /animal) insult. On a comparative weight basis it was consistently more potent than α_1-PI [209].

β-Lactams

In the early studies exploring the interaction of lactams with mammalian serine proteinases, a single β-lactam derivative (11a) had been reported to react with α-chymotrypsin to form a very stable acyl-enzyme, that is, it was an alternate-substrate inhibitor [204]. However, after this report more than



(11a) (11b) R = H (11c) R = CH_2Ph (11d) R = H (11e) R = t-Bu

Figure 2.11. β-lactam inhibitors of serine proteinases.

twenty years elapsed before it was reported by a group at Merck that cephalosporins, which are well-known, β-lactam, acylating inhibitors of bacterial serine proteinases, could be modified to become potent time-dependent inhibitors of HLE [210]. Perhaps inspired by the fact that many different classes of β-lactams are used clinically for the acute treatment of bacterial infections, the Merck group has broadly explored the *in vitro* and *in vivo* SAR, physical properties, mechanism and toxicity profiles of several series of β-lactam inhibitors and inactivators of HLE.

The impetus for the newer work was the observation that benzyl clavulanate (11c) is a time-dependent inactivator of HLE (IC_{50} = 5 μM) whereas clavulanic acid (11b) is inactive [211, 212]. This finding led to the hypothesis that, since HLE is an endopeptidase whereas the bacterial serine proteinases are carboxypeptidases, 'quench(ing) the negative charge that the β-lactam antibiotics normally require' might yield HLE inhibitors. For synthetic reasons the group at Merck decided to use 7-aminocephalosporanic acids (11d) for most of their initial SAR studies. In contrast to the result with clavulanic acid, conversion of the cephalosporin 2'-carboxyl group in (11d) to an ester (11e) was insufficent to transform the compound to an HLE inhibitor.

It was discovered that replacement of the 7-β-NH_2 substituent in (11e) with a small, preferably electron-withdrawing 7-α-substituent such as methoxy (16-1, *Table 2.16*) or chloro (16-2), resulted in inhibition of HLE. Furthermore, oxidation of the ring sulphide either to a (β)-sulphoxide (16-3) or a sulphone (16-4 or -5) resulted in step-wise increases in potency. Both of these changes, in the 7-substituent and the oxidation state of sulphur, are known to enhance the chemical reactivity of the β-lactam ring. It was hypothesized that the reason a 7-α-substituent is preferred is that only in this orientation can a C^7-substituent fit in the S_1 site of the enzyme. The detailed SAR studies on this series revealed that the nature of the 3'-leaving group had a significant effect on *in vitro* potency (not shown) and that potency could be correlated to physical properties in a regression analysis using a

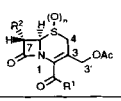
combination of electronic and steric parameters [213]. Also, it was determined that substitution at C^4 had an effect on potency (not shown) which could range from significant increases to decreases, depending on the C^2-acyl substituent [214].

Although these cephalosporin C^2-esters were potent *in vitro* inhibitors of HLE, their ester group is hydrolytically unstable and such compounds are rapidly degraded in blood. As a means of increasing the stability of these compounds replacement of the C^2-ester with an amide group was explored, for example, (16-6 or 16-7). Although the *in vitro* potency decreased for these two compounds, the change to an amide did result in increased stability and (16-7) was sufficiently active *in vivo* (see discussion below) that it was chosen for extensive study as a potential aerosol drug [215].

The mechanism of action of these β-lactam inhibitors has been the object of several studies, which have indicated a complex situation that appears to vary depending on the C^7-substituent [216–218]. An X-ray crystallographic study using PPE indicated that (16-5) was a mechanism-activated inhibitor which could make two covalent links to the enzyme (see *Figure 2.12*) [216]. Unlike the previously discussed heterocyclic inhibitors, some of the cephems showed a remarkable degree of enzyme selectivity against a variety of other proteolytic enzymes [215].

Although the C^2-amide cephalosporin derivatives showed increased stability as compared with the C^2-esters, they were still somewhat unstable. As an example, (16-7) had $t_{1/2}$ values for hydrolysis at pH 8 (25°C) and for

Table 2.16. INHIBITION OF HLE BY CEPHEM β-LACTAMS[a]

Compound	 *16-0*			$IC_{50}(\mu M)$	$k_{obs}/[I]$ $(M^{-1}\,s^{-1})$	*Ref.*
	R^1	R^2	n			
(16-1)	O-*t*-Bu	OMe	0	10.0	ND	211
(16-2)	O-*t*-Bu	Cl	0	2.0	ND	211
(16-3)	O-*t*-Bu	OMe	1	4.0	ND	211
(16-4)	O-*t*-Bu	OMe	2	1.0	19,000	211
(16-5)	O-*t*-Bu	Cl	2	ND	161,000	211
(16-6)	NH-*t*-Bu	OMe	2	ND	2,200	215
(16-7)		OMe	2	ND	3,800	215

[a]See footnote 'e' in *Table 2.1*.

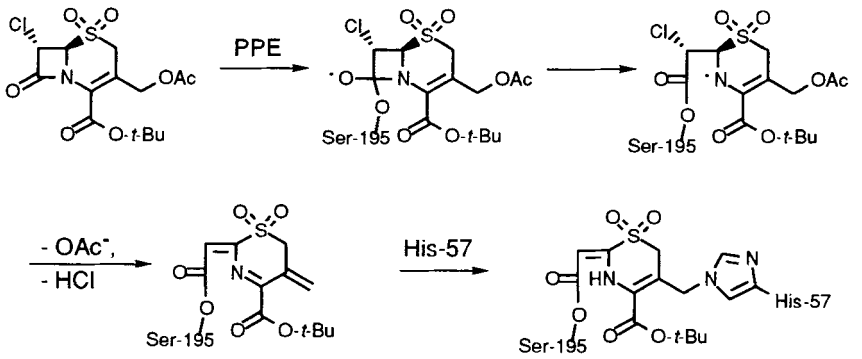


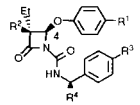
Figure 2.12. Mechanism of enzyme inactivation by (some) β-lactam inhibitors.

degradation in rat blood, of 22 h and < 10 min., respectively [219]. Therefore, these cephalosporins lacked systemic activity and were effective *in vivo* only following i.t. administration. Due to the desire to develop an orally active compound, other series of β-lactam inhibitors were explored, and some azetidinones (17-0) were discovered to have promising levels of *in vitro* activity and hydrolytic stability.

One of the first azetidinones, (17-1), showed modest activity *in vitro*, combined with much improved hydrolytic stability ($t_{1/2}$ > 80 h). Incorporation of a second C^3-ethyl substituent yielded (17-2), which was two orders of magnitude more stable to hydrolysis than (17-1) and which showed no degradation in rat blood after 12 h. Furthermore, this compound showed activity *in vivo* in hamsters, following oral dosing. Additional additive increases in the *in vitro* potency of these azetidinones was achieved by: (a) replacement of the 4-*p*-hydroxybenzoic acid substituent, for example, (17-1, *Table 2.17*) with a 4-*p*-hydroxyphenylacetic acid group, for example, to give (17-3); (b) introduction of a *p*-methyl group to the benzyl amide, for example, (17-4); and (c) introduction of a (*R*)-propyl chain at the α-carbon of the benzyl amide to give (17-5) [220]. This last compound is very effective at inhibiting HLE, showing ~25% of the activity of α_1-PI (on a molar basis), along with exceptional selectivity.

In vivo studies for many of the Merck β-lactam inactivators have been reported. In the cephem series, these reports have focused on the i.t. profile of (16-7) which was determined to possess the best overall properties for an aerosol drug candidate. This compound had an ED_{50} of 5 μg /animal in the AHA model when dosed i.t. 30 min. prior to HLE challenge. It showed sustained duration of action in the AHA model with a t_{50} (the pre-insult time

Table 2.17. INHIBITION OF HLE BY AZETIDINONES[a]

Compound	 *17-0*				k_{inact}/K_i $(M^{-1}\,s^{-1})$	$k_{obs}/[I]$ $(M^{-1}\,s^{-1})$	*Ref.*
	R^1	R^2	R^3	R^4			
(17-1)	CO_2H	H	H	H	ND	2,480	219
(17-2)	CO_2H	Et	H	H	ND	1,500	219
(17-3)	CH_2CO_2H	Et	H	H	ND	3,200	219
(17-4)	CH_2CO_2H	Et	Me	H	188,000	4,500	219
(17-5)	CH_2CO_2H	Et	Me	Pr	622,000	ND	220

[a]See footnote 'e' in *Table 2.1.*

interval needed to achieve 50% inhibition at a given dose of drug) of 185 min. when using an i.t. dose of 200 mg/ animal prior to HLE challenge. In the azetidinone series, reports have focused on the oral profile of (17-5) (L680,833). This compound has an ED_{50} of 1.5 mg/kg in the AHA model when administered orally 5 h before challenge with HLE [221].

CLINICAL STUDIES

The vast majority of the clinical trials of HLE inhibitors have utilized α_1-PI. These studies have been designed to demonstrate the feasibility of replacement therapy, the safety of acute and chronic administration and the pharmacokinetic profile of exogenously applied protein. Most of these questions were approached in the late 1960s and late 1970s. The first human trials to demonstrate the feasibility of supplemental α_1-PI administration were reported in 1969 [222]. At that time, serum α_1-PI deficiency was a well recognized trait in man, and the association between decreased serum proteinase inhibitor levels and the increase risk for the development of COPD had been made. However, the biological mechanism responsible for the deficiency was not known. In an attempt to determine whether increased catabolism was responsible for the observed low circulating levels of α_1-PI, human protease inhibitor was isolated from healthy, α_1-PI sufficient individuals, iodinated and administered to both homozygous deficient and homozygous sufficient volunteers. The elimination half-life of radiolabled

α_1-PI was found to range from 3 to 6 days in normal volunteers and 5 to 6.5 days in deficient individuals. The lack of major differences in elimination rates led the investigators to conclude that the defect responsible for α_1-PI deficiency was not due to catabolism, but more likely was due to defects in either synthesis or secretion of the protein. These results were confirmed and extended in 1970 [223] in studies which also demonstrated that intravenously administered α_1-PI could augment sputum elastase-inhibitory capacity. Additional studies in the late 1970s further examined the catabolism and elimination of α_1-PI in humans. It was observed that there were no major differences in the rates of elimination of either piMM (normal) of piZZ type α_1-PI [224] and that desialylated α_1-PI was eliminated at a much faster rate ($t_{1/2}$ of approximately 5 min.) than was the intact inhibitor ($t_{1/2}$ of 4 to 5 days) [225]. These early studies, in addition to demonstrating the lack of differences in metabolism of deficient individuals or in the type of α_1-PI found in deficient individuals, also showed the potential feasibility of replacement therapy to correct α_1-PI deficiency.

Studies reported in 1981 [226] demonstrated that weekly administration of α_1-PI could augment and maintain the elastase inhibitor capacity (EIC) of the lung without significant safety concerns. A more detailed pharmacokinetic and dose-ranging study using a commercially prepared α_1-PI preparation was reported in 1987 [227]. These investigators determined that a dose of 60 mg provided circulating levels of α_1-PI that were in excess of the threshold level of 80 mg/deciliter [as determined from previous epidemiological studies] in piZZ patients one week post-infusion. Serum and bronchoalveolar lavage fluid (BALF) EIC levels both increased dramatically and were maintained throughout the study. The serum half-life of the material infused did not differ over the course of the study from that determined after the first infusion. A similar study [228] demonstrated that once-monthly infusions of 250 mg/kg doses of α_1-PI were capable of maintaining circulating levels of protease inhibitor and enhanced the EIC of BALF. As in the previous studies, no adverse clinical reactions due to therapy were reported. These investigations demonstrated the feasibility of long term administration of α_1-PI as supplemental therapy for α_1-PI deficient patients and effectively set the doses that have been routinely used in clinical evaluation of the commercial product.

The feasibility of aerosol administration of α_1-PI has also been examined [229]. α_1-PI was administered by inhalation at a dose of 100 mg every 12 h for 7 days to a group of α_1-PI deficient patients. After a single inhalation dose of inhibitor, BALF α_1-PI levels increased approximately 6-fold relative to pre-therapy levels. After one week of twice daily therapy, BALF levels were approximately 21-fold higher than at the initiation of the trials. The

therapy was well tolerated by the patients and no adverse reactions were observed during the study.

Recombinant α_1-PI produced in yeast has also been clinically evaluated. This material is essentially identical to α_1-PI purified from human serum with respect to its protein sequence and its ability to inhibit neutrophil elastase; however, it contains no carbohydrates. As such, one would suspect that the elimination rate following intravenous administration would be similar to that described for desialylated α_1-PI as described above [225]. In monkeys r-α_1-PI disappeared from circulation with a half-life of 69 $\pm$ 3 min. compared to a half-life of 2.4 $\pm$ 0.2 days for α_1-PI isolated from human serum [230]. Rapid elimination following parenteral administration does not, however, preclude aerosol administration of the recombinant material. Aerosol administration of yeast-produced r-α_1-PI, in doses ranging from 10 to 200 mg, resulted in a dose-dependent elevation of both antigenic recombinant α_1-PI and EIC of BALF from α_1-PI deficient individuals. Four hours after administration of a 200 mg dose of recombinant α_1-PI, EIC was increased 40-fold over pre-treatment levels. Twenty-four hours post-dose, levels had declined to a 5-fold elevation relative to base-line levels. Modest increases of serum α_1-PI levels were observed 24 h post-inhalation of the 200 mg dose [231]. In this study, no adverse clinical reactions or evidence of sensitization to recombinant α_1-PI were reported.

These and other studies have amply demonstrated the safety and feasibility of supplementing or replacing endogenous levels of α_1-PI by exogenously applied inhibitor, either partially purified from human serum or produced through recombinant techniques. However, there are as yet no published studies reporting that inhibitor replacement slows or halts the progression of emphysema. It is of interest, that none of the α_1-PI replacement studies of reasonable duration, i.e. 3 to 6 months or longer, discuss the effect of replacement therapy on levels of biochemical markers associated with elastase activity *in vivo* [232].

α_1-PI is also being clinically evaluated in diseases other than those associated with the well-characterized, inhibitor-deficiency state. In cystic fibrosis, the inflammation of the lungs and airways are associated with very high levels of free neutrophil elastase. In theory, inhibition of the excessive protease burden may lead to a resolution of some of the symptoms associated with this lethal disease. In one clinical trial, intravenous and aerosol therapy with α_1-PI has been evaluated [233]. Weekly intravenous administration of up to 120 mg/kg partially purified α_1-PI for four weeks failed to depress the burden of elastase found in the lungs of cystic fibrosis patients. However, aerosol administration of 1.5 to 3.0 mg/kg of the same

inhibitor every 12 h for one week resulted in inhibition of BALF elastase activity. At BALF α_1-PI levels of 8 μmol/liter, there was complete inhibition of elastase in the lavage fluid. Additionally, polymorphonuclear leukocytes exposed to pre-therapy BALF exhibited decreased killing of *Pseudomonas aeruginosa*. Post-treatment BALF did not impair neutrophil killing of Pseudomonas. These encouraging results suggest that longer term studies with α_1-PI or other elastase inhibitors may lead to a reduction of the pulmonary symptoms associated with cystic fibrosis.

α_1-PI has also been evaluated in an open-label trial in intractable atopic dermatitis [234]. The authors hypothesized that the inflammation associated with atopic dermatitis might be due to a local increase of serine proteases and/or decrease of endogenous inhibitors in the presence of normal global levels of protease inhibitors. They selected six patients with atopic dermatitis that had failed to respond to high-potency topical steroids to evaluate the topical effect of exogenously applied α_1-PI. Patients were initially (days 1 to 20) treated on alternate days with 6 ml of a 20 mg/ml solution of α_1-PI in either vinyl gloves (5 of 6 patients) or an op-site wound kit for 2 consecutive hours followed by application of 1% α_1-PI cream. This sequence was repeated three times daily. Nocturnal treatment consisted of continuous application (8 h) of the aqueous solution in occlusive covering. From days 21 to 90, five of the six patients were treated with a maintenance therapy of a 5% α_1-PI cream, with or without the addition of a topical steroid. Serial photographs were taken to document patient progress. Symptoms were also recorded. Four of six patients reported an almost immediate relief of pain and pruritus and an increased range of motion following initiation of therapy. The suggestion was made that the immediate effect of therapy was related to the ability of α_1-PI to inhibit kallikreins, proteases responsible for the production of kinins such as bradykinin. All six patients responded favourably to therapy as indicated by decreased pain and pruritus and extended range of motion as described above, and by decreased erythema, increased exfoliation, re-epithelization and healing of fissures and ulcers. None of the five patients entering maintenance therapy (either with or without steroids) relapsed during the study. All of these patients relapsed within 20 to 40 days of discontinuance of maintenance therapy. One of the six patients received no maintenance therapy and did not relapse. Recognizing the limitations of this small, open-label trial, this is the first indication of actual therapeutic efficacy (relief of symptoms) reported for α_1-PI and is certainly worthy of further studies.

Similar, but less extensive, studies of secretory leukocyte protease inhibitor (SLPI) have also been performed. Most of the pharmacokinetic evaluation of this material has been performed in animals [235, 236]. In

man, the elimination half-life of intravenously injected ^{125}I-SLPI has been estimated to be approximately 2 h [237]. SLPI has been clinically evaluated in cystic fibrosis patients [238]. Aerosol administration of 100 mg SLPI, over a 25 min. period, to cystic fibrosis patients resulted in a significant increase of SLPI recoverable in BALF and a corresponding decrease of active HLE. The average decrease of free elastase for the entire group of 20 patients was 68%. The free elastase in BALF fluid of cystic fibrosis patients has previously been demonstrated to induce bronchial epithelial cells to express the gene for the inflammatory mediator and neutrophil chemoattractant, IL-8 [239]. Those results were confirmed in this study. Pre-therapy BALF induced a marked expression of the gene for IL-8 in resting epithelial cells. In marked contrast, BALF recovered post-SLPI therapy was much less effective as an inducer of the IL-8 gene. The *in vitro* effects were replicated *in vivo*. Lavagable IL-8 levels fell from a pre-therapy level of 21.7 nM to a post-SLPI level of 10.7 nM. Lavagable neutrophils were reduced by approximately 45%, consistent with the 52% reduction of lavagable IL-8. The data obtained in this study also demonstrated a significant, linear relationship between lavagable free elastase and IL-8 levels.

ACKNOWLEDGEMENTS

The authors thank Drs. Frederick J. Brown and Chris A. Veale for their valuable suggestions and comments on this review.

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Progress in Medicinal Chemistry – Vol. 31, edited by G.P. Ellis and D.K. Luscombe

3 The Medicinal Chemistry of the Azido Group

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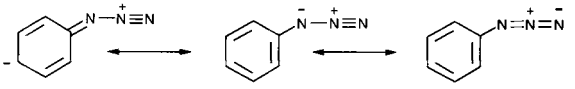
INTRODUCTION

The first documented synthesis of an azido-substituted organic compound appeared in 1864, when Griess synthesized phenyl azide (azidobenzene) (1) from benzenediazonium tribromide and ammonia [1]. Subsequent recognition of the wide synthetic utility of reactions involving azide intermediates has led to a resurgence of interest in azide chemistry, particularly over the last three decades, resulting in the publication of several specialized monographs and a number of comprehensive texts on the subject [2–4]. However, the azido substituent has only recently gained some recognition as a novel pharmacophore in medicinal chemistry, due largely to the emergence of AZT* (zidovudine) (2) as an effective drug for the treatment of acquired immune deficiency syndrome (AIDS). Although azides also

*The following abbreviations are used in this review: ADP, adenosine diphosphate; AIDS, acquired immune deficiency syndrome; AMT, 3′-amino-3′-deoxythymidine; Ara-A, adenosine arabinoside; Ara-C, cytosine arabinoside; Ara-U, uridine arabinoside; AZAL, L-3-azidoalanine; AzddC, 3′-azido-2′,3′-dideoxycytidine; AzddG, 3′-azido-2′,3′-dideoxyguanosine; AzddMeC 3′-azido-2′,3′-dideoxy-5-methylcytidine; AzddU, 3′-azido-2′,3′-dideoxyuridine; azidoMeIQx, 2-azido-3,8-dimethylimidazo[4,5-*f*]quinoxaline; AZT (zidovudine), 3′-azido-3′-deoxythymidine (note that 'deoxy-' at the 2′-position is implicit in the name 'thymidine', making this a 2′,3′-dideoxynucleoside); BOC, *t*-butyloxycarbonyl; DDMP, metoprine; DNCB, 1-chloro-2,4-dinitrobenzene; DHFR, dihydrofolate reductase; DI, diazepam-insensitive; DS, diazepam-sensitive; *DTE, dithioerythritol*; DTIC, *dacarbazine*; DTT, dithiothreitol; FDA, Food and Drugs Administration; FNPA, 4-fluoro-3-nitrophenyl azide; GABA, γ-aminobutyric acid; GSH, glutathione; HMT, histamine-*N*-methyltransferase; HSV, herpes simplex virus; IP, imidazo[4,5-*b*]pyridine; IQ, 2-amino-3-methylimidazo[4,5-*f*]quinoline; MAO, monoamine oxidase; MAP, *m*-aminopyrimethamine; MBP, methylbenzoprim; MNP, *m*-nitropyrimethamine; MTX, methotrexate; MZP, *m*-azidopyrimethamine; MZPES, *m*-azidopyrimethamine ethanesulphonate; N_3UDP, 2′azido-2′-deoxyuridine-5′-diphosphate; NADPH, β-dihydronicotinamide-adenine dinucleotide phosphate; NADP, β-nicotinamide-adenine dinucleotide phosphate; NBC, 4-nitrobenzyl chloride; NCI, National Cancer Institute; NIH, National Institutes of Health; OT, oncogene transformed; PAF, platelet-activating factor, PBZ, phenylbutazone; PET, positron emission tomography; PI, phosphatidylinositol; PLA_1, phospholipase A_1; PPi, inorganic pyrophosphate; PYM, pyrimethamine; QSAR, quantitative structure-activity relationship; RNR, ribonucleotide reductase; RT, reverse transcriptase; SAR, *structure-activity relationship*; UDPGT, uridine 5′-diphosphoglucuronosyltransferase; WT, wild-type.

have an established role in biochemistry as photoaffinity reagents, this aspect of the biology of the azido group is beyond the scope of the current review, and the reader is referred to appropriate monographs on the subject [5, 6].



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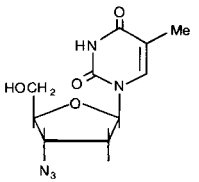

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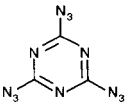


(3)

It is of interest to note that the popularity of the azido group as a photoactivatable substituent is derived, in part, from the synthetic accessibility of azido-substituted derivatives of the natural substrate or drug, and the ease with which retention of high receptor affinity, a prerequisite in the design of such molecules, may be achieved. Indeed, on more than one occasion, the introduction of an azido group for the purpose of conducting photolabelling experiments has afforded a derivative with intrinsically interesting biological properties compared with the parent compound. In this chapter physico-chemical and biological properties of the azido functionality of interest to the medicinal chemist are highlighted, and examples of biologically active azides appearing in the scientific literature up to early 1993 are discussed.

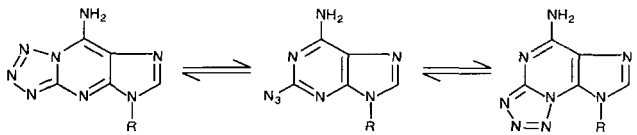
BACKGROUND

PHYSICO-CHEMICAL PROPERTIES OF THE AZIDO GROUP

Once thought to exist as a cyclic structure, the azido group in organic azides is now known to be linear, this being first demonstrated with the determination of the X-ray crystal structure of cyanuric triazide (3) [7].

However, some evidence to support the existence of a cyclic form of azide ion has been adduced more recently [8]. Not surprisingly, the azide ion (N_3) is symmetrical in nature but this symmetry is perturbed in organic azides, the unsubstituted nitrogens having the shorter bond distance, and azides are usually represented by a number of resonance hybrid structures as shown for phenyl azide (1). For methyl azide (MeN_3), the substituent is disposed to the three nitrogens at an angle of 120° although this is reduced slightly in phenyl azide which exhibits a C–N–N bond angle of 114°. When an azido group is directly attached to a carbon double bonded to a nitrogen (4a, X = NH), the imidoyl azide exists in tautomeric equilibrium with the corresponding tetrazole (4b, X = NH), although the equilibrium invariably favours the tetrazole. Acyl azides (4a, X = O) are thought to exist entirely in the open chain form while only cyclic tautomers (4b, X = S) of thiocarbonyl azides (4a, X = S) have been observed.

(4a) (4b)



(5b) (5a) (5c)

Azides and tetrazoles usually equilibrate slowly at room temperature and the position of the equilibrium, which varies with temperature and solvent, is also susceptible to steric and electronic effects, electron-withdrawing substituents favouring the azide tautomer. The azinyl-azide/tetrazole tautomeric equilibrium, which has been extensively investigated and reviewed [9, 10], also arises when the C = N double bond comprises part of a heterocyclic ring, as exemplified by 2-azidoadenosine (5a, R = β-D-ribofuranosyl) synthesized for use, as the diphosphate, as a photoaffinity reagent for the ADP receptor [11]. The azide was found to equilibrate with one or both of the two possible tetrazoles (5b and 5c) with an azide:tetrazole ratio of 1:1 at pH 7.0, and in contrast to the azide, which was readily photolyzed at 270nm, the tetrazole tautomer was resistant to photolysis. Azides that exist predominantly as tetrazoles may thus exhibit different

chemical and biological properties from their linear counterparts, and this must be taken into consideration when the possibility of tautomeric equilibration exists.

The azido group, together with several other functionalities including the cyano and isocyanato groups, exhibits some properties in common with the halogens, and as a consequence, compounds containing the azido substituent have been termed 'pseudohalides' [12] since they often exhibit physico-chemical characteristics akin to the corresponding halide [13]. Thus, in terms of approximate size, polarity and electronic character the azido group resembles a bromo substituent as shown in *Table 3.1*, and like the halides, it exerts a negative inductive effect (-I), and a positive mesomeric effect (+M) on an aromatic ring, activating the ring towards electrophilic attack. Similarities between the azido substituent and the halogens have alerted some investigators to the possibility of utilising the azido group as a halide bioisostere in drug design, and examples of this will be referred to later.

Although azides and halides have some chemical reactions in common, for example, as leaving groups in nucleophilic substitution reactions, unlike halides the intrinsic reactivity of the azido group both to nucleophiles and electrophiles enables this substituent to participate in a wide variety of reactions often resulting in loss of nitrogen. Indeed, the propensity of certain azides to decompose violently on heating, subjection to mechanical shock or exposure to certain chemicals, is legend. However, although some

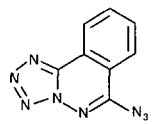
Table 3.1. SIMILARITIES BETWEEN PHENYL AZIDE (1) AND BROMOBENZENE [2, 13, 15]

Property	*Phenyl azide*	*Bromobenzene*
Boiling Point (°C)	189[a]	156
Dipole Moment	1.44D	1.54D
Molar Refraction	10.2B	8.88B
Molar Magnetic Rotation (15°C)	14.77	14.51
Log P (π)	0.46	0.86
σ_m	0.37	0.39
σ_p	0.08	0.23
σ_p^+	−0.54	0.15

[a]Calculated.

azides, including the highly sensitive cyanuric triazide (3), are dangerously unstable and should be handled with extreme caution [14], it is accepted that the stability of organic azides increases with the size of the carbon skeleton to which the azido group is attached, and it has been suggested that as a general rule, compounds for which the ratio (C + O)/N is less than 3 should be regarded as potentially explosive [15]. The photolytic decomposition of azides with loss of nitrogen and the generation of several highly reactive electrophilic species, including singlet and triplet nitrenes, forms the basis for the use of azides as photoaffinity reagents.

Only one naturally occurring azide has been reported to date. 6-Azidotetrazolo[5,1-*a*]phthalazine (6) was isolated, by chloroform extraction and subsequent chromatographic purification, from unialgal cultures of *Gymnodinium breve* (*Ptychodiscus brevis*), a dinoflagellate responsible for the production of toxic red tides along the Gulf Coast of Florida [16]. The structure of this extraordinary secondary metabolite was determined by



(6)

X-ray crystallography and the azide was found to be icthyotoxic, with an LD_{100} of 0.4 μg/ml, although the mechanism of toxicity does not appear to have been studied. It is of interest to note that in accordance with the general rule described above, this compound (molecular formula $C_8H_4N_8$) should be regarded as potentially explosive!

TOXICITY AND BIOLOGICAL ACTIVITY OF INORGANIC AZIDE

Hydrazoic acid and salts

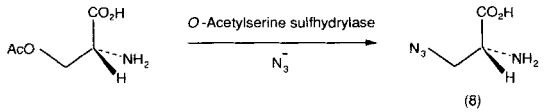
The pharmacology, toxicity and mutagenic properties of inorganic azide have been extensively investigated, and since detailed reviews cover the subject up to 1982 [17, 18], the following section will focus on important developments in the interim. Hydrazoic acid (HN_3) and its salts are extremely toxic and some nine fatalities have been reported in the last decade, following the accidential or deliberate ingestion of sodium azide [19-21]. The acute toxicity of hydrazoic acid is comparable with that of hydrogen cyanide and the symptoms of poisoning, which include irritation of the eyes and mucous membranes, intense headache, dyspnoea and

hypotension, may be delayed following inhalation, although no reports of irreversible effects after chronic exposure to low concentrations of the gas have been documented to date. Aside from the acute toxicity of hydrazoic acid and sodium azide, the principal pharmacological activity of these compounds appears to be vasodilation, and sodium azide ('smite') has been employed clinically for the control of hypertension [18, 22]. Indeed, although its use has been discontinued due to the hazardous nature of the salt, three hypertensive patients were maintained on small oral doses of sodium azide for as long as a year with no apparent adverse effects [23].

Mutagenic properties of inorganic azide

The mutagenicity of azide ion has been known for over forty years [24]. Millimolar concentrations of sodium azide were found to significantly increase the number of antibiotic-resistant mutant *Staphylococcus aureus* or *Micrococcus pyogenes* in culture, and this was attributed to an accumulation of hydrogen peroxide in bacterial cells due to the inhibition of catalase and peroxidase [24, 25]. Sodium azide mutagenesis was subsequently observed in a number of higher plants including barley, and the deleterious accumulation of hydrogen peroxide was again deemed responsible until Owais, Kleinhofs and coworkers, in a series of elegant experiments, adduced evidence to suggest that peroxide accumulation was not a valid explanation for the mutagenic activity of azide [17]. Having isolated a substance excreted by *Salmonella typhimurium* treated with sodium azide which proved mutagenic in the Ames test [26], they confirmed an earlier report that L-cysteine inhibited azide mutagenicity [27], and also found that while bacterial production of the mutagen was suppressed when L-cysteine or L-cystine was added to the growth medium, the mutagenicity of the extracted component was unaffected by subsequent addition of these amino acids [28]. These results added to the growing body of evidence that azide was not a mutagen per se but required metabolic activation to the ultimate genotoxic agent.

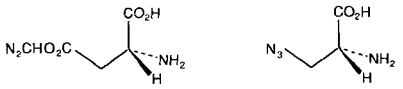
Researchers in Warsaw reported that, in the absence of exogenous *O*-acetylserine, *S. typhimurium* mutant strains lacking serine transacetylase were not susceptible to azide mutagenicity implying that *O*-acetylserine was necessary for the conversion of azide to a mutagenic metabolite. They demonstrated subsequently that mutant bacteria deficient in *O*-acetylserine sulfhydrylase (EC 4.2.99.8), an enzyme required for the biosynthesis of L-cysteine from *O*-acetylserine and inorganic sulphide, were not susceptible to azide mutagenicity and concluded that this enzyme was essential for the synthesis of a mutagenic derivative of azide, possibly the established



(8)

Figure 3.1. The biosynthesis of azidoalanine (8) from inorganic azide and O-acetylserine in S. typhimurium.

mutagen azaserine (7) [29]. Subsequent studies eventually led to the purification and characterization of the azide metabolite isolated from *S. typhimurium* as L-3-azidoalanine (AZAL) (8) [30]. Thus, in a reaction catalysed by *O*-acetylserine sulfhydrylase, inorganic azide reacts with *O*-acetylserine to produce 3-azidoalanine and acetate (*Figure 3.1*), and the structure of this metabolite was shown to be identical with authentic material synthesized by two independent groups [31, 32].

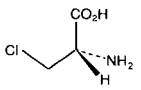


Owais and Gharaibeh have recently demonstrated conclusively the involvement of *O*-acetylserine sulfhydrylase by cloning the gene for the enzyme from wild type *E. coli* into a mutant strain deficient in this enzyme, whereupon the mutant strain was able to convert azide into a metabolite that was mutagenic to *S. typhimurium* [33]. However, it is difficult to reconcile the chemical stability of AZAL with a direct genotoxic effect and AZAL appears to be a proximate mutagen requiring further bioactivation to the ultimate mutagenic agent. Azide ion and AZAL are nearly equally mutagenic *in vivo*, while the enantiomeric D-AZAL has a greatly reduced mutagenic potential and this is consistent with the subsequent rate-limiting enzymic conversion of AZAL to an as yet unidentified ultimate mutagenic agent [32]. A detailed account of the work leading to the identification of AZAL has been published by Owais and Kleinhofs [34].

The nature of the ultimate mutagen remains to be established but, in bacteria, a deficiency in excision-repair processes greatly enhances azide mutagenicity, implying the generation of a direct DNA lesion [35]. Unlike a number of mutagenic azido analogues including azidoethidium and azidoacridine (see page 133), the mutagenicity of azide is not potentiated by light [34], and thus if azidoalanine activation entails analogous nitrene formation and subsequent covalent interaction with DNA, this must arise

via a metabolic process. Interestingly, azide appears to be non-mutagenic and non-carcinogenic in animals [36], with only marginal genotoxicity to cultured mammalian cells being reported [37, 38], and consequently the use of sodium azide as a mutagenic agent in mutational plant breeding has been recommended as less hazardous than other established mutagens [39]. Since mammals utilize a different metabolic pathway for the biosynthesis of cysteine, the lack of azide-induced mutagenicity was at first ascribed to the absence of *O*-acetylserine sulfhydrylase in these cells [40]. However, a group at the University of Texas found that Chinese hamster V79 cells expressed *O*-acetylserine sulfhydrylase, although the isolated enzyme, intact V79 Chinese hamster cells or normal human skin fibroblasts in culture each on its own was unable to convert azide to appreciable quantities of a mutagenic product [41]. The putative mutagen AZAL was later found to exhibit only slight genotoxicity when incubated directly with these cell lines [42], and it appears that, in contrast to bacteria and some plant systems, either AZAL is poorly transported into mammalian cells, or further metabolism to the ultimate mutagen does not arise. Alternatively, it is possible that efficient repair of any azide-induced genetic damage occurs or that mammalian cells metabolize azide *via* an alternative route to a non-toxic product. Some evidence that cellular transport may account for this differential toxicity was very recently provided by Raicu and Mixich, who observed increased genotoxicity following the exposure of heteroploid human cells to sodium azide incorporated into liposomes, and who attribute this to the efficient delivery of azide ion into the cells [43]. The cellular uptake of AZAL by mammalian cells does not appear to have been studied, and it is also possible that poor cellular transport compared with bacteria may account for the observed lack of toxicity.

In order to further delineate the role of AZAL in azide mutagenicity, a number of structure-activity studies have been undertaken and the results are summarized in *Table 3.2*. The close structural analogues 3-chloroalanine (9) and 3-cyanoalanine (10) were both found to be non-mutagenic [30], underlining the importance of the azido substituent for genotoxicity. Interestingly, *O*-acetylserine sulfhydrylase is quite catholic in the substrates



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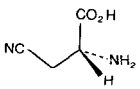
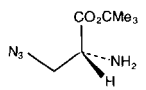


(10)

it will accept and these include 1,2,4-triazole and cyanide [44], the latter giving rise to a toxic but non-mutagenic metabolite, thought to be

3-cyanoalanine, in *S. typhimurium* [45]. LaVelle and Mangold in Connecticut investigated the role of the amino and carboxyl groups of AZAL in mutagenesis by the synthesis of 3-azidopropionic acid ($N_3[CH_3]_2CO_2H$) which lacks an amino substituent, and analogues protected at the carboxyl and/or amino groups as the *t*-butyl ester and *t*-butyloxycarbonyl (BOC) derivatives (11–13), respectively [46]. They found that mutagenicity to *S. typhimurium* in the Ames test was greatly reduced as compared with that of racemic AZAL, on removing the amino substituent (to give 3-azidopropionic acid) or blocking it as a lipophilic derivative (12) and (13), while derivatization of the carboxyl group as an ester had no effect on activity and (11) was equipotent with AZAL. The very weak mutagenic activity exhibited by (13), where both amino and carboxyl groups are blocked, is consistent with an earlier observation that ethylamine is essentially non-mutagenic [30].



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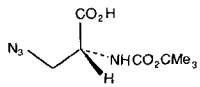

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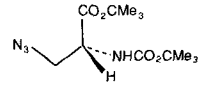


(13)

From these results, it was proposed that the presence of a free amino group is an absolute prerequisite for activity, but that the role of the carboxyl substituent is less certain. The nearly identical mutagenic potencies of AZAL and the lipophilic ester (11) also argues against the proposition that the lack of increased potency of AZAL over azide ion is derived from cellular exclusion of the former, since the highly lipophilic ester should readily gain ingress to cells. Owais and co-workers also compared the mutagenic potency of AZAL with 2-azidoethylamine ($H_2NCH_2CH_2N_3$) and two homologues, 3-azidopropylamine ($H_2N[CH_2]_3N_3$) and 4-azidobutylamine ($H_2N[CH_2]_4N_3$) [30]. 2-Azidoethylamine proved to be a somewhat weaker mutagen than AZAL; this suggests that a carboxyl substituent may be necessary for maximal activity, and mutagenic activity was found to decline rapidly with increasing carbon chain length, 4-azidobutylamine being virtually devoid of activity (*Table 3.2*).

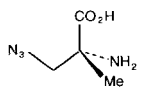
These structure-activity studies, when taken in tandem with reports that the activity of AZAL resides predominantly in the L-isomer, strongly support the further enantioselective metabolic bioactivation of AZAL, possibly by L-amino acid metabolizing enzymes, to a mutagenic product. More recently, the Connecticut group have attempted to gain further insight into possible mechanisms of AZAL activation by studying the effects of homologation and α-methyl substitution of the amino acid [47]. α-

Table 3.2. MUTAGENICITY OF AZIDOALANINE AND RELATED COMPOUNDS IN REPAIR-DEFICIENT BASE SUBSTITUTION STRAINS OF S. *TYPHIMURIUM* (TA1530 AND TA100) [30]

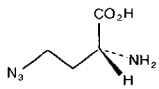
Compound [a]	*Number of His+ revertants/0.1ml/plate* [b]	
	TA1530	*TA100*
Control	24	150
Sodium azide	2370	1950
3-Azidoalanine (8)	2612	2418
2-Azidoethylamine	820	620
3-Azidopropylamine	46	148
4-Azidobutylamine	30	140
2-Bromoethylamine	520	152
3-Bromopropionic Acid	506	625
3-Chloroalanine (9)	26	146

[a]All compounds evaluated in the absence of light at a final concentration of 0.5 mM;
[b]Average of duplicate plates.

Methylazidoalanine (14) was approximately 3 orders of magnitude less potent than AZAL in inducing *S. typhimurium* mutants in the Ames test, and the authors suggest that this reduction in mutagenicity may correlate with the inability of the analogue to participate in enzyme-catalysed deamination reactions, thereby indicating a possible role for this metabolic pathway in the bioactivation of AZAL. The likelihood of an AZAL catabolite entering into the Krebs cycle was also evaluated with azidoacetic acid ($N_3CH_2CO_2H$), a putative substrate for this metabolic pathway, but the compound was significantly less mutagenic than AZAL, implying that it does not play a role in AZAL mutagenicity. That potent mutagenic activity is not confined to AZAL was convincingly demonstrated by the direct homologue 2-amino-4-azidobutanoic acid (15), which proved to be nearly 4-fold more potent than the parent compound and also exhibited enantioselective mutagenicity, the L-isomer proving the more active enantiomer. The mutagenicity of other azido-substituted amino-acids or



(14)



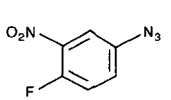
(15)

peptides does not appear to have been explored and further investigations are warranted. A group at Farmitalia Carlo Erba has found recently that batches of hormone-like peptides synthesized by the azide-coupling method contained traces of water-soluble impurities mutagenic in the Ames test [48]. The mutagenic component(s) were not identified and although the authors speculate that traces of sodium azide may have been responsible, the possible involvement of small quantities of adventitious azidoamino-acids or azidopeptides cannot be discounted.

BIOLOGICAL ACTIVITY OF ORGANIC AZIDES

Mutagenicity

Aside from the above studies with azidoalanine and closely related compounds, the mutagenic potential of other organic azides does not appear to have been thoroughly investigated. Phenyl azide (1) and a number of substituted derivatives were found to be non-mutagenic to several species of *S. typhimurium* and to V79 Chinese hamster cells in the presence of various metabolizing enzymes [49]. At relatively high concentrations, 4-fluoro-3-nitrophenyl azide (16) and 2,4-dinitrophenyl azide (17) were both unusual in proving mutagenic to all five of the commonly used tester strains of mutant *S. typhimurium* in the Ames test, without requiring metabolic activation, indicating that these compounds might represent useful standard mutagens [50]. Owais *et al.* found that of a series of azidoalkylphthalimides (18) (n = 1–4) synthesized as intermediates for azidoalanine mutagenicity studies, only the relatively unstable azidomethylphthalimide (n = 1) exhibited significant mutagenic activity in *S. typhimurium*, and it was suggested that a decomposition product may have been responsible [30]. Indeed, the possibility of facile release and subsequent metabolic activation of azide ion from activated azides such as (17), must be considered when ascribing genotoxicity to organic azides.



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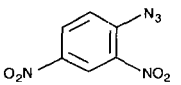

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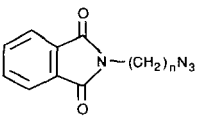
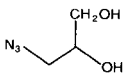


(18)

In addition to its toxicity, sodium azide appears to be quite a capricious mutagen when utilized for the induction of desired mutants in industrially useful micro-organisms, particularly yeasts and fungi, due perhaps to an

inability of some of these organisms to convert azide to azidoalanine [17]. Juricek and colleagues in Prague observed that a series of azido-sugars and azido-alcohols evaluated as potential alternative agents for mutation breeding, exhibited similar mutagenic activity to azide ion [51, 52]. The most potent of these compounds, 3-azido-1,2-propanediol (3-azidoglycerol) (19) was found to be active in micro-organisms insensitive to sodium azide, exhibiting mutagenicity comparable with that of alkylating agents in a wide spectrum of bacteria, yeast and higher plants. Unlike these agents, 3-azidoglycerol was ineffective in inducing chromosomal aberrations in human peripheral lymphocytes *in vitro* [53, 54]. Interestingly, although 3-azidoglycerol resembled sodium azide in producing light-independent base-substitution but not frameshift mutations, in *S. typhimurium* and *E. coli*, the mutagenic activity of the former was not influenced by addition of cysteine to the medium, indicating that the mutagenicity of 3-azidoglycerol does not involve metabolic conversion to 3-azidoalanine. Moreover, unlike sodium azide, addition of glycerol to the medium reduced the mutagenicity of 3-azidoglycerol in a competitive manner, although whether the parent compound competes with the azido-alcohol for cellular uptake or as a substrate for subsequent metabolic activation has not been established [52]. Sodium azide, 3-azidoglycerol and a number of other azido-alcohols and azido-sugars exhibit similar mutation patterns, and it is possible that these compounds undergo metabolic activation to a common ultimate mutagen, although this does not appear to be 3-azidoalanine. The ketoacid pyruvate represents a common product of the metabolism of alanine, glycerol and glucose, and the Prague group propose that azidopyruvate (20) may be implicated in the mutagenesis of azides, although this remains to be confirmed.



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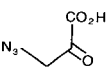
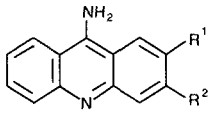


(20)

Perhaps the most intensively studied mutagenic organic azides are analogues of the DNA-interactive agents acridine and ethidium and their derivatives. The broad spectrum of biological activity exhibited by acridines is thought to arise from their ability to intercalate DNA [55, 56], and the genotoxic activity of these compounds has been extensively reviewed [57]. Azido-substituted analogues of aminoacridines and ethidium have been widely utilized as photoaffinity probes to elucidate sites of DNA intercalation, with the pioneering studies of Yielding and colleagues being among the most notable [58, 59]. Structure–mutagenicity relationships were

determined (in the Ames test) for twenty azidoacridine analogues synthesized as potential photoaffinity reagents for the frameshift mutagen 9-aminoacridine (aminacrine) (21) [58]. Mutagenicity and toxicity in the Ames *S. typhimurium* strains was compared with that of a large number of non azido-substituted acridines in the dark, and while the azidoacridines were generally more toxic and mutagenic than the parent compounds, analogous patterns of activity were observed regarding the positioning of substituents on the acridine ring. Thus, 2-azido-9-aminoacridine (22), the most promising of the analogues, exhibited similar biological properties to 9-aminoacridine in the absence of light. Upon light activation, the 2-azido derivative was more effective as a frameshift mutagen than 9-aminoacridine, although, interestingly, photolysis of the 3-azido-9-aminoacridine congener (23) significantly reduced mutagenicity, and this was attributed to competing solvolysis of the resultant nitrene with consequent introduction of aromatic ring substituents, for example, 3-hydroxylamino (24), incompatible with the intercalation process.



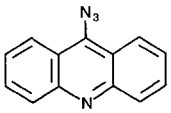
(21) $R^1 = H$; $R^2 = H$

(22) $R^1 = N_3$; $R^2 = H$

(23) $R^1 = H$; $R^2 = N_3$

(24) $R^1 = H$; $R^2 = NHOH$

In contrast to the 9-aminoacridine analogues, which were predominantly frameshift mutagens regardless of the nature of other substituents including azido groups, 9-azidoacridine (25) and its derivatives were found to be base-substitution mutagens [58, 60] with or without exposure to light. Very similar activity was also seen upon methylation of the ring nitrogen of 9-azidoacridine to afford 9-azido-10-methylacridinium chloride (26). Mair and Stevens examined the chemical properties of azidoacridine derivatives and found that protonation or methylation of the ring nitrogen of 9-azidoacridine rendered the 9-azido group highly labile to nucleophiles [61]. They suggested that DNA-intercalation and subsequent covalent 'heteroarylation' of a suitably positioned nucleophile might represent an



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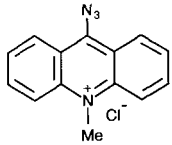
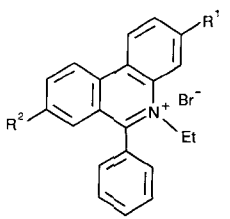


(26)

alternative mechanism of mutagenesis for the compound, although no direct evidence has been offered to support this hypothesis.

Some interesting results have been obtained with azido analogues of ethidium bromide (27), an intercalator requiring prior metabolic activation by mammalian enzymes in order to exhibit frameshift mutagenicity in the Ames test [62]. Replacing either the 3- or 8-amino substituents by an azido group afforded derivatives (28), (29) that unlike the parent compound, were potent frameshift mutagens requiring photoactivation but not conversion to an active metabolite [59]. Conducting mutagenicity studies in the absence of light, or irradiating the compounds prior to incubation produced few mutations, which is consistent with the photolytic generation of a nitrene and subsequent covalent modification of DNA. The 3,8-diazidoethidium derivative (30) retained some mutagenic activity on photolysis but was approximately 40–100 times less active than either of the monoazido-substituted compounds, while monoazides (31), (32) lacking an amino group were feeble mutagens. The authors propose that at least one amino function is necessary to facilitate the correct interpositioning of the ethidium analogue into DNA during intercalation. Subsequent nitrene generation then results in a covalent binding to DNA with the phenanthridium ring in a biologically-active orientation. The possibility that an azido substituent or a photolysis product may also function to orientate the molecule into the mutagenic conformation, albeit less efficiently than an amino group, may explain the weaker mutagenic activity of the diazide (30).



(27) $R^1 = NH_2$ $R^2 = NH_2$

(28) $R^1 = N_3$ $R^2 = NH_2$

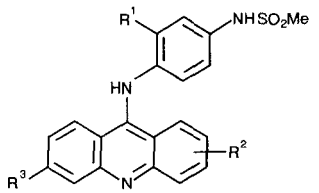
(29) $R^1 = NH_2$ $R^2 = N_3$

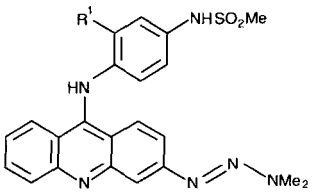
(30) $R^1 = N_3$ $R^2 = N_3$

(31) $R^1 = H$ $R^2 = N_3$

(32) $R^1 = N_3$ $R^2 = H$

One other notable series of azidoacridine analogues is derived from the antitumour agent amsacrine (33), a 9-anilinoacridine developed by Cain and colleagues in Auckland, and clinically useful in the treatment of acute leukaemia [63, 64]. The drug is thought to act primarily by stabilizing the cleavable complex formed between the enzyme DNA-topoisomerase II and DNA [65], and, in keeping with many other acridine derivatives, amsacrine and related compounds are mutagenic in a number of prokaryotic and eukaryotic cellular systems [57]. In a structure–activity study involving over 500 anilinoacridines, the Auckland group reported that 3-azidoamsacrine (34) exhibited a very similar spectrum of antitumour activity and mutagenicity to the parent compound (33) [66]. Interestingly, the same group had previously synthesized 3-azidoamsacrine as a result of promising antitumour activity observed with a number of lipophilic 3-(3,3-dialkyltriazeno)acridine analogues of amsacrine, as exemplified by the dimethyltriazenes (38) and (39) [67]. The investigators wished to evaluate a 3-substituent structurally very similar to the triazeno group but lacking alkyl substitution on the unsaturated three nitrogen chain, and the azides (34) and (36) were found to exhibit excellent antitumour activity *in vivo* against L1210 tumour-bearing mice, suggesting that an azido functionality may represent a useful bioisostere for the triazeno group.





(33) R^1 = OMe R^2 = H R^3 = H

(34) R^1 = OMe R^2 = 3-N_3 R^3 = H

(35) R^1 = H R^2 = 2-N_3 R^3 = H

(36) R^1 = H R^2 = 3-N_3 R^3 = H

(37) R^1 = H R^2 = 2-N_3 R^3 = 6-N_3

(38) R^1 = H

(39) R^1 = OMe

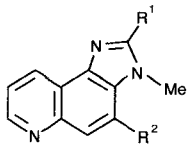
As part of a study to establish 3-azidoamsacrine (34) as a viable photoaffinity probe for amsacrine-DNA interactions, the cytotoxic and mutagenic properties of this compound were investigated utilizing mammalian cell-mutation assays with and without light activation [68]. Not surprisingly, 3-azidoamsacrine was cytotoxic to V79 Chinese hamster cells

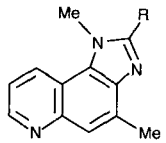
in vitro and the cytotoxic effects were increased on photoactivation. However, unlike the parent compound (33) which is an effective inducer of chromosomal breaks in the V79 cell-mutation assay [69], the azide (34) was not mutagenic to these cells regardless of photoactivation. This contrasts with the results for analogous studies conducted with the murine L5178Y leukaemia cell-mutation assay. In this cell line, amsacrine and its 3-azido analogue exhibited similar patterns of mutagenicity with and without light activation, the cytotoxicity of the 3-azido analogue being potentiated following exposure to light. Subsequent photoaffinity labelling studies conducted with (34) indicated that cytotoxic and, in some cases, mutagenic activity results from covalent binding to DNA, possibly via photoadduct formation with the 2- and 6-amino substituents of deoxyguanosine and deoxyadenosine, respectively.

In a very recent investigation, the mutagenicity and DNA-strand scission potential of 3-azidoamsacrine (34) and ten analogues bearing azido groups at various positions was assessed in *S. typhimurium*, with and without photoactivation [70]. Of the azides evaluated, compounds (35) and (36) induced massive DNA single-strand cleavage on irradiation, as did the highly potent 2,6-diazidoacridine (37), and although the expected alkylation of DNA by (34) was detected, DNA cleavage was not apparent for this derivative. A possible important role for the azido substituent in enhancing DNA-binding or association with topoisomerase II, was inferred from the observation that, in the absence of light, analogues (35) and (36) were both more mutagenic than the corresponding parent compounds lacking an azido group.

An interesting application of aryl azides as biochemical probes for elucidating the mechanism of action of mutagenic and carcinogenic arylamines has been reported. The bioactivation of such arylamines and nitroarenes is thought to proceed via a common proximate mutagen, probably an arylhydroxylamine derivative, which subsequently suffers further metabolism to a highly electrophilic arylnitrenium ion believed to represent the ultimate DNA-reactive species [71]. However, mechanistic studies with arylhydroxylamine derivatives have been impeded by the difficulty of synthesis and inherent instability of these compounds. Thus, the possibility of utilizing aryl azides as reagents for the direct non-metabolic generation, upon photolysis, of the ultimate mutagenic species without recourse to the arylhydroxylamine intermediates, was investigated. Wild and Dirr [72] discovered that 2-azido-3-methylimidazo[4,5-*f*]quinoline (azido-IQ) (43), an azido derivative of the carcinogen 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) (41) found in fried meat, elicited a high frequency of mutations in *S. typhimurium* on photoactivation, and that rates of

azido-IQ consumption and induction of mutations were related to photolysis times. However, the number of mutations was only reduced by approximately a half when the study was conducted in an arylhydroxylamine-resistant *S. typhimurium* strain, and this was in contrast to the parent arylamine (41) which, as expected, was non-mutagenic in this resistant strain. Residual genotoxicity was attributed to a highly DNA-reactive species, clearly not the arylhydroxylamine (42), and probably a nitrene or nitrenium ion generated directly by photolysis of the azide.





(40) $R^1 = NO_2$ $R^2 = H$

(41) $R^1 = NH_2$ $R^2 = H$

(42) $R^1 = NHOH$ $R^2 = H$

(43) $R^1 = N_3$ $R^2 = H$

(44) $R^1 = NO_2$ $R^2 = Me$

(45) $R^1 = NH_2$ $R^2 = Me$

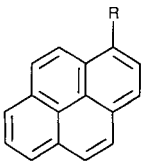
(46) $R^1 = N_3$ $R^2 = Me$

(47) $R = NO_2$

(48) $R = NH_2$

(49) $R = N_3$

In a subsequent photolysis study, the DNA-binding and mutagenic activity of a series of azides was found to correlate closely with that established for the corresponding arylamines and nitroarenes, with high mutagenic activity being observed for azido-IQ (43), a closely related derivative 2-azido-3,8-dimethylimidazo[4,5-*f*]quinoxaline (azido-MeIQx) (46) and the polycyclic arene 1-azidopyrene (52) [73]. In common with their nitro (40), (47), (50) and amino (41), (48), (51) derivatives, the azides also induced frame shift mutations in *S. typhimurium*, and DNA-adducts obtained in *S. typhimurium* or calf thymus DNA after photolysis of azido-IQ (43) or 1-azidopyrene (52), were indistinguishable from those generated metabolically by the corresponding Nitro-IQ or 1-nitropyrene derivatives (40), (50). From these results, the authors conclude that the same highly reactive species, probably a nitrenium ion, is formed by the metabolism of arylamines and nitroarenes and the photolysis of aryl azides, and propose that azides represent convenient precursors for the single-step photo-generation of the ultimate mutagens of arylamines and nitroarenes.



(50) R = NO_2

(51) R = NH_2

(52) R = N_3

This 'azide-nitrene' strategy was employed in studies to determine SARs for a series of azide derivatives including analogues of aminoimidazoarenes related to IQ (41) [74, 75]. The rank order of mutagenic potency for the azides was again found to parallel closely that reported for the corresponding amino- and nitro-imidazoarenes, and these results strongly suggested that the mutagenic activity of arylamines, nitroarenes and aryl azides involves the formation of a common nitrene/nitrenium ion as the ultimate mutagen. Structure–activity studies demonstrated that while simple aryl azides were only weakly mutagenic, activity was increased for fused-ring aromatic azides, and maximal genotoxicity was observed for imidazoarenes with a quinoline ring system bearing an *N*-methyl substituent adjacent to the azido function, as for azido-IQ (43) and the isomeric azido-isoIQ (49) (*Table 3.3*). These results were interpreted in terms of differences in the stability, and hence electrophilic character, of the nitrenium ion postulated as the ultimate DNA-reactive species. The heterocyclic imidazoaromatic ring of azido-IQ (43) and azido-isoIQ (49) offers excellent resonance stabilization of the nitrenium ion via efficient delocalization of the positive charge and the imidazole-methyl group further stabilizes the system by preventing deprotonation. Very recently, the results of quantitative structure-activity relationships (QSARs), determined for nineteen aryl azides, demonstrate that mutagenicity is directly related to the stability of the nitrenium ion, and that azide mutagenicity correlates with the mutagenic activity of the corresponding arylamine [76]. Thus, the efficient stabilization of imidazoylnitrenium ions compared with simple arylnitrenium species may account for the extraordinary mutagenic and carcinogenic activity exhibited by these compounds.

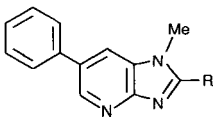
More recently, the mechanism of genotoxicity of the aminoimidazoarene (PhIP, 54), the most prominent of the heterocyclic arylamines found in fried meat products, was studied in comparison with the corresponding nitro (53) and azido (55) derivatives [77]. PhIP is a potent mutagen in mammalian cell

Table 3.3. MUTAGENIC ACTIVITY OF ARYL AZIDES IN HYDROXYLAMINE-RESISTANT (TA98/1,8-DNP$_6$)S.*TYPHIMURIUM*.[a]

Compound	*Mutagenic activity (revertants/nmol)*	*Ref.*
Phenyl azide (1)	<0.04	73
1-Azidonaphthalene	2.4 ± 0.2[b]	73
2-Azidonaphthalene	16.1 ± 0.5	73
4-Azidodiphenyl	20.3 ± 1.8	73
2-Azidofluorene	695 ± 22	73
6-Azidochrysene	936 ± 61	73
1-Azidopyrene (52)	3470 ± 165	73
Azido-IQ (43)	13400 ± 670	72
Azido-methyl-IQ	39200 ± 2930	72
Azido-isoIQ (49)	44000 ± 2420	74

[a]Plates irradiated with near ultraviolet light (365–366nm);
[b]S.D.

lines but is only weakly mutagenic in *S. typhimurium* and by employing the azide-nitrene technique, it was possible to explain these differences in terms of atypical metabolic activation of PhIP and nitro-PhIP in bacterial and mammalian cells.



(53) R = NO_2

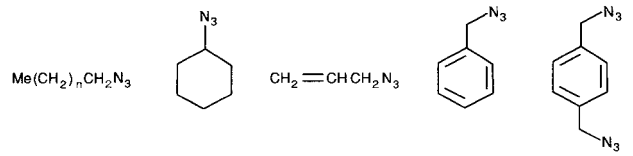
(54) R = NH_2

(55) R = N_3

Enzyme inhibitory activity

The well-established utility of sodium azide as a biochemical reagent stems from its activity as an inhibitor of numerous metal-containing enzymes, including catalase, peroxidase and the haem-containing proteins involved in

cellular respiration [17, 78]. However, there is a paucity of information regarding the enzyme-inhibitory activity of organic azides, and in this section the few compounds reportedly active by virtue of possessing an azido group are reviewed. Potential therapeutic azides which exert their pharmacological effects through the inhibition of enzymes will be discussed later (see page 160). The possibility that the grazing habits of camels might result in their ingestion of azido-substituted herbicides, prompted a group in Jordan to evaluate the interaction of a number of alkyl azides with glutathione *S*-transferase from the liver, lung and kidney of these animals [79]. Inhibitory activity was determined in a spectrophotometric assay at 340 nm, where rates of enzyme-catalysed conjugation of either 1-chloro-2,4-dinitrobenzene (DNCB) or 4-nitrobenzyl chloride (NBC) with glutathione (GSH), were measured in the presence of varying concentrations of inhibitor. The inhibitors employed for these studies were the simple homologous alkyl azides (56–60) where n = 1-5, cyclohexyl azide (61), allyl azide (62), benzyl azide (63) and 1,4-bis(azidomethyl)benzene (64).



(56-60) (61) (62) (63) (64)

All of the azides investigated were time-dependent inhibitors at millimolar concentrations and the inhibition was reversible in each case, with hepatic glutathione *S*-transferase proving the most sensitive enzyme. Inhibitor potency appears to depend upon the substrate employed, *n*-heptyl and allyl azides (60) and (62) being the most potent with NBC, and *n*-butyl and *n*-hexyl azide (57) and (59) when DNCB was included in the assay. Kinetic studies, where the GSH and DNCB concentrations were independently varied, indicated that compounds (61),(63) and (64) were non-competitive inhibitors, while allyl azide (62) and the *n*-alkyl azides (56)–(60) inhibited the enzyme in a competitive manner. From these observations, the authors speculate that, in a process reminiscent of that known to occur with alkyl and aryl halides, glutathione *S*-transferase may catalyse the conjugation of azides with GSH *in vivo*.

In the course of studies directed towards the development of photoaffinity labels for monoamine oxidase (MAO) isozymes, Shih and colleagues found that 4-fluoro-3-nitrophenyl azide (FNPA) (16) exhibited some interesting properties as an inhibitor of this enzyme [80]. MAO catalyzes the oxidative

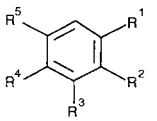
deamination of a wide range of endogenous amines and xenobiotics, with two principal enzyme sub-types, MAO-A and MAO-B, being characterized largely on the basis of substrate specificity and selective inhibition. The effects of FNPA on MAO-A and MAO-B isolated from rat brain cortex were determined, in the dark, in assays employing radiolabelled serotonin or phenethylamine, respectively as substrates, and the aryl azide was found to inhibit both enzymes competitively with MAO-B being slightly more sensitive ($K_i = 0.78$ μM) than MAO-A ($K_i = 3.0$ μM). This selectivity of inhibition was also observed in competitive binding studies with the irreversible inhibitors deprenyl (MAO-B) and clorgyline (MAO-A). FNPA completely prevented the inactivation of MAO-B by deprenyl but only partially protected the MAO-A isozyme from the effect of clorgyline at the same concentration, implying that FNPA competes efficiently with deprenyl for binding at the active site of MAO-B. Interestingly, photolysis of the FNPA–MAO-B complex resulted in a time-dependent irreversible inactivation of the enzyme in a reaction presumed to entail covalent labelling of the active site, since addition of a high concentration of the MAO-B substrate phenethylamine effectively abolished the reaction. Moreover, the photodependent labelling of MAO-B by FNPA decreased radiolabelling of the enzyme by [^{3}H]-pargyline, an irreversible inhibitor of MAO-B, by occupying the pargyline binding site. In contrast, significant photodependent inactivation of MAO-A by FNPA was not evident under identical experimental conditions, inferring that the substrate/inhibitor binding domains of the two isozymes differ considerably with regard to their interaction with FNPA.

In order to delineate the mechanism of selective photolabelling of MAO-B by FNPA, the same investigators synthesized a series of structurally related aryl derivatives (65)–(70) and studied their effects on the two classes of enzyme [81]. The structures of the derivatives, together with their competitive K_i values determined against rat brain cortex MAO-A and MAO-B, are summarized in *Table 3.4*. All of the derivatives resembled FNPA in being singularly less potent inhibitors of MAO-A than MAO-B, and it is clear that a fluoro substituent is not a prerequisite for activity with neither the presence nor the position of the group influencing potency. Crucially, the results demonstrate the requirement of an azido group for maximal inhibitory activity against either isozyme, as demonstrated by the dramatic reduction of potency on its removal, as in 2-fluoronitrobenzene (69) or substitution by an amino group in 4-fluoro-3-nitroaniline (70). The authors also report that 2,4-dinitrofluorobenzene, where an electron-withdrawing nitro group replaces the azido substituent of FNPA, is also a much less potent MAO inhibitor. They propose that the azido group does

not function simply to decrease the electron density of the aromatic ring but rather contributes directly in the binding of FNPA and related compounds to MAO-B.

The inhibitory potency of phenyl azide (1) is also noteworthy in that the K_i values against MAO-A (230 μM) and MAO-B (18 μM) approximately parallel the respective literature K_m values for phenethylamine (278 μM and 20 μM), suggesting that phenethylamine and phenyl azide may associate with the same relative affinities and bind prefentially to MAO-B as substrate and inhibitor, respectively. More controversially, it is proposed that FNPA and related aryl azides are structurally similar to phenethylamine and inhibit MAO because the azido group resembles the 2-aminoethyl side-chain of the substrate. The presence of a nitro substituent is also clearly important for binding to MAO, probably by making an electrostatic interaction with the enzyme, and steric effects are also in evidence from the relative inhibitory activities of the three isomeric nitrophenyl azides (66)–(68) against the two enzyme sub-types. One possible explanation offered for the inefficient photodependent incorporation of FNPA and derivatives into MAO-A is that the azide-binding region is much less constrained than that of MAO-B, enabling the reactive nitrene formed on

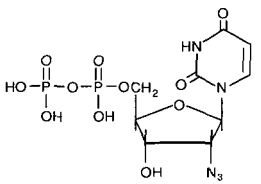
Table 3.4. INHIBITORY ACTIVITY OF FNPA (16) AND DERIVATIVES AGAINST MAO-A AND MAO-B [81]



Compd.	R^1	R^2	R^3	R^4	R^5	*Inhibition constant (K_i) (μM)*	
						MAO-A	*MAO-B*
1	N_3	H	H	H	H	230	18
16	N_3	H	NO_2	F	H	3	0.8
65	N_3	F	H	H	NO_2	–	0.6
66	N_3	H	NO_2	H	H	9	0.4
67	N_3	H	H	NO_2	H	10	0.3
68	N_3	NO_2	H	H	H	9	3.5
69	NO_2	F	H	H	H	31	5
70	NH_2	H	NO_2	F	H	30	20

irradiation to react with solvent rather than covalently labelling the enzyme. This hypothesis is supported by the results of the structure–activity studies, where the azide-binding domain of MAO-A exhibits a greater tolerance to the steric effect of a nitro group than that of MAO-B, consistent with a more exposed active site. Thus, 2-nitrophenyl azide (68) is approximately an order of magnitude less potent than the isomeric 3- or 4-nitrophenyl azides (66) and (67) against MAO-B, but all three compounds are equipotent against MAO-A. In common with phenyl azide (1), the 2-nitro derivative (68) failed to react photodependently with MAO-B.

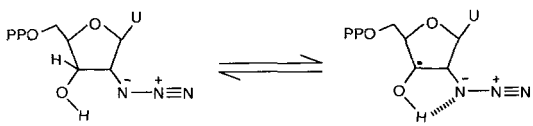
An unprecedented example of the application of an organic azide as an enzyme inhibitor derives from the elegant studies of Stubbe and coworkers at MIT, who have investigated the mechanism of action of ribonucleotide reductase (RNR) using several mechanism-based inhibitors including 2′-azido-2′-deoxyuridine-5′-diphosphate (N_3UDP) (71) [82]. RNR plays a



(71)

crucial role in DNA biosynthesis by catalyzing the conversion of ribonucleoside diphosphates into the corresponding 3′-deoxyribonucleotide diphosphates, in a complex reaction which, for mammalian and certain bacterial systems, appears to involve the intermediacy of a tyrosyl radical [83]. The crucial step of the reaction is thought to involve hydrogen abstraction, by the protein radical, from the 3′-position of the ribonucleoside thus generating a radical adjacent to the 3′-hydroxyl substituent.

Incubation of *E. coli* RNR with N_3UDP results in rapid enzyme inactivation via a covalent modification, and destruction of the tyrosyl



(71)

Figure 3.2. Stabilization of the putative radical of N_3UDP (71) by intramolecular hydrogen bonding [85].

radical with the concomitant production of nitrogen, uracil and inorganic pyrophosphate. The 2′-azido group appears to function as an intramolecular radical trap, possibly facilitated by hydrogen bonding via the 3′-OH as shown in *Figure 3.2* [84], generating a new radical species which subsequently decomposes with inactivation of the enzyme, and although the precise mechanism by which this occurs remains to be fully elucidated, two possible fragmentation pathways have been proposed (*Figure 3.3*). Thus, either loss of nitrogen or generation of an azide radical followed by β-elimination of uracil and inorganic pyrophosphate (PPi) would furnish unsaturated ketones (71a) or (71b), respectively, either of which could then alkylate and inactivate the enzyme [85]. Clearly, N_3-UDP has proven an invaluable probe of the mechanism of action of RNR, an enzyme regarded as a potentially useful chemotherapeutic target in proliferative and

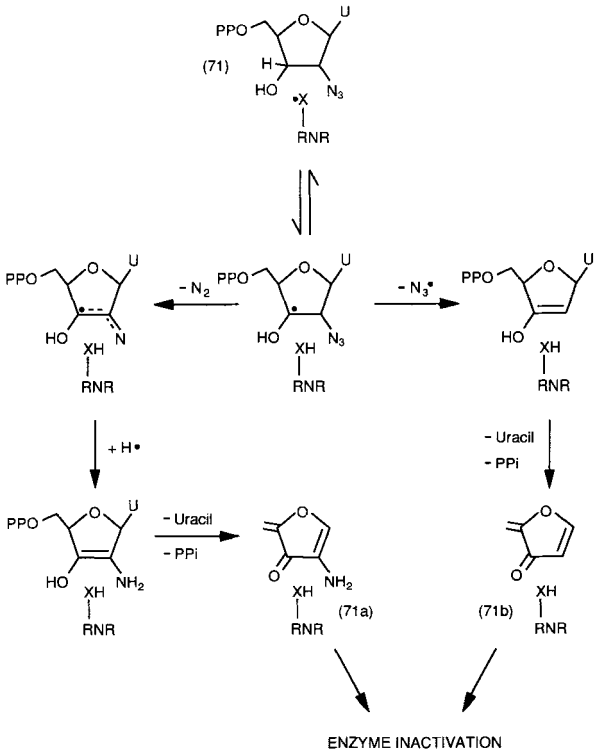


Figure 3.3. Possible mechanisms for the inactivation of RNR by N3UDP (71). (Reproduced with permission from reference [84]; copyright 1990, The Royal Society of Chemistry.)

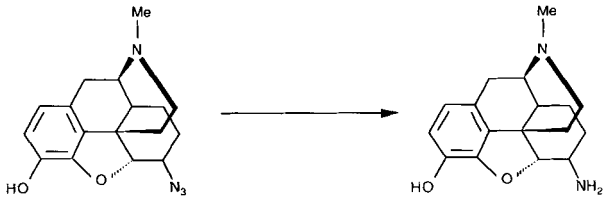
infectious disease, and future examples of the application of the azido group in analogous mechanistic studies are likely. It is of interest to note in the context of the following discussion on the non-metabolic bioreduction of azides (page 155), that the active-site of RNR contains two redox thiols, and it has been suggested that reduction of the azido group of N_3-UDP may be thiol-mediated [84].

The metabolism of organic azides

Little is known about the metabolism of organic azides and of the toxicological and pharmacokinetic implications of introducing an azido substituent into a drug molecule. From a chemical reactivity viewpoint, azides are extremely resistant to oxidation and readily susceptible to reduction to the corresponding amine [15], and one might expect this to be paralleled in biological systems, with biotransformation pathways involving reduction of the azido substituent predominating. Hawkins suggested that, in a manner analogous to that observed for azo compounds, intestinal flora may play a role in the reduction of azides [86], although this remains to be established. One of the earliest reported investigations of the metabolism of an azide, was by Knoll and co-workers in 1975 [87], and concerned the *in vitro* and *in vivo* stability of the alkyl azide azidomorphine (6-deoxy-6-azidodihydroisomorphine) (72). They found that, in common with morphine and related opiates, the semi-synthetic alkaloid suffered *N*-demethylation in rat liver microsome preparations and was extensively conjugated to the glucuronide in the rat. However, infrared spectroscopic analysis of radiochromatographically purified azidomorphine metabolites, extracted from rat liver microsomes after incubation with [7,8-^{3}H]azidomorphine, demonstrated the persistence of an absorption band at 2120 cm^{-1} characteristic of the azido group and implying that metabolism of the azido function had not occurred to a significant degree. A similar result was also obtained with metabolites isolated, after 24 h, from the urine of rats injected subcutaneously with azidomorphine, and the authors concluded that the azido group in this compound was strongly resistant to metabolism.

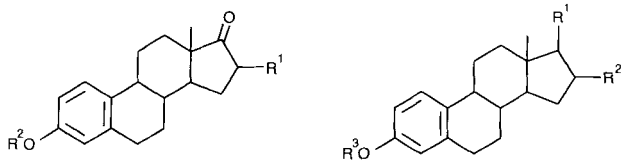
However, in a subsequent study with a human subject, who received a single subcutaneous dose of azidomorphine, small quantities of a metabolite characterized as 6-deoxy-6-β-aminodihydroisomorphine ('aminomorphine', 73) were identified in the urine by g.l.c-m.s [88]. This metabolite proved identical with an authentic sample prepared by the reduction of azidomorphine with sodium borohydride, and was detected at a urinary concentration approximately 10-fold lower than that of the parent compound and its conjugates. Although only conducted with one subject,

this investigation was one of the first to demonstrate the metabolic reduction of an azide to the corresponding amine, and also served to highlight differences between man and rat regarding azide metabolism, since aminomorphine was not observed in the urine of rats following administration of (72).



(72) (73)

The unusual and unprecedented biotransformation of an azido-substituted compound has been reported by Schumann who investigated the preclinical metabolic fate of the steroid derivative 16α-azido-3-methoxyestra-1,3,5(10)-trien-17-one (STS-267) (74), a novel hypocholesterolaemic and fertility inhibitory drug with reduced hepatotoxicity, in perfused rat isolated liver preparations [89]. Four metabolites were isolated by t.l.c following perfusion of [^{3}H]-STS-267, together with significant quantities of unchanged steroid and a number of unidentified water-soluble products. The four metabolites were characterized, by comparison with authentic compounds, as the 3-*O*-demethylated steroid (75), the 3,17-β-diol (76) and, most interestingly, the two 16β-azido epimers (77) and (78). The author proposes that the major isolated metabolite (78) arises from the parent compound (74) by 17-carbonyl reduction and epimerization of the 16-azido substituent with or without 3-*O*-demethylation. Subsequent biological studies with the parent steroid or its metabolites do not appear to have been published.



(74) R^1 = alpha-N_3 R^2 = Me

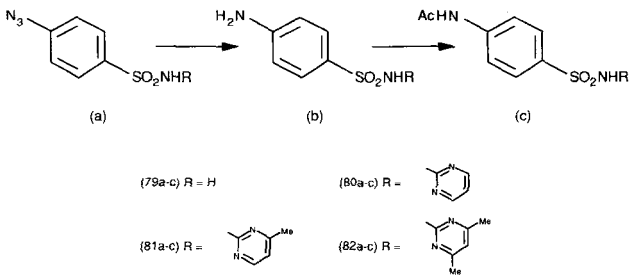
(75) R^1 = alpha-N_3 R^2 = H

(76) R^1 = beta-OH R^2 = alpha-N_3 R^3 = H

(77) R^1 = beta-OH R^2 = beta-N_3 R^3 = Me

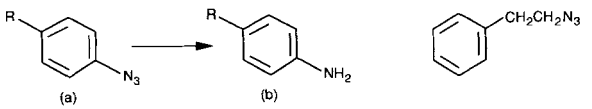
(78) R^1 = beta-OH R^2 = beta-N_3 R^3 = H

The most in-depth studies regarding the biological fate of the azido group in aromatic azides have been conducted by Stevens and colleagues at Aston University, who were among the first to propose a possible role for azides as prodrug modifications of amines [90, 91]. In order to establish whether inactivation, via azide bioreduction, might compromise the activity of a series of aryl azides synthesized as potential antitumour radioaffinity reagents, and discussed below, the *in vivo* metabolism of several azidobenzenesulphonamides (79a)–(82a) was evaluated in rats [90, 92]. Examination, by extraction and t.l.c analysis, of urine collected over 24 h after intraperitoneal administration of the compounds in 10% acetone/arachis oil, revealed the presence of significant quantities of the corresponding aminobenzenesulphonamides (79b)–(82b) together with unchanged starting materials and several unidentified metabolites. Further conjugative metabolism of three of the aminobenzenesulphonamides (79b)–(81b) to their *N*-acetyl derivatives (79c)–(81c) was also evident, although, surprisingly acetamidosulphadimidine (82c) was not detected in the urine of rats after administration of (82a) [92]. Structural assignment was constrained to a chromatographic comparison with authentic material, and the urinary concentrations of each azidosulphonamide metabolite were not quantified. Nevertheless, these results confirmed that metabolism of the azido substituent in aryl azides to the corresponding arylamine could occur, an observation that alerted the Aston group to the possibility of utilizing this biotransformation in the design of short-acting antitumour antifolates. Azidosulphonamides could conceivably serve as lipophilic prodrugs of the parent sulphonamides but this has not been investigated, and the antibacterial activity of these compounds remains to be reported.



As part of our studies with azido-substituted drugs, we investigated further the nature of the biotransformation of the azido substituent, and the effect upon metabolism of other functionalities on the aryl ring, using phenyl azide (1a) and a series of simple 4-substituted aromatic azides

(83a)–(86a) as model substrates [93]. The required aryl azides were synthesized in excellent yields from the corresponding aniline derivatives (83b)–(86b) by the well established diazotisation/azidation method [94], and these were incubated with mouse hepatic microsome preparations under aerobic or anaerobic conditions, metabolite analyses being performed by h.p.l.c. The alkyl azide, phenethyl azide (88) was also included in these studies for comparative purposes. Interestingly, under aerobic conditions, no metabolism of the azido group was observed for any of the compounds studied, although phenyl azide (1a) and 4-methoxyphenyl azide (84a) both underwent oxidative metabolism to the same product identified as 4-azidophenol (87a). Under a nitrogen atmosphere, the aromatic azido compounds (1a) and (84a), and the alkyl azide (88) were not metabolized to a detectable degree, whereas 4-chloro- and 4-cyano-phenyl azide (83a) and (85a) underwent slow metabolic conversion to metabolites that co-chromatographed with authentic samples of the expected arylamines (83b) and (85b), respectively. By contrast, under these anaerobic conditions 4-nitrophenyl azide (86a) was rapidly and exclusively metabolized to 4-nitroaniline (86b) at a linear rate some 20-fold that observed for (83a) or (85a), the rate of amine formation being dependent upon substrate concentration and proportional to the concentration of microsomal protein.



(1a-b) R = H
(83a-b) R = Cl
(84a-b) R = OMe
(85a-b) R = CN
(86a-b) R = NO_2
(87a) R = OH

In order to elucidate further the nature of this biotransformation, the effect of varying incubation conditions upon the rate of metabolism of compound (86a) was studied as shown in *Table 3.5*. We found that heat denaturation of the microsomal preparation or omission of an NADPH-generating system effectively abolished metabolism, implying that bioreduction of the azido group was enzyme-catalyzed rather than arising through an alternative mechanism involving a simple chemical reaction with endogenous thiols, as discussed below. The enzyme responsible for this bioreduction was not identified, but the dependence on NADPH, absence of dramatic inhibition by carbon monoxide and high sensitivity to oxygen was

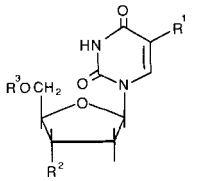
Table 3.5. EFFECT OF MICROSOMAL INCUBATION CONDITIONS ON THE RATE OF FORMATION OF *p*-NITROANILINE (86b) FROM *p*-NITROPHENYL AZIDE (86a) [93]

Incubation conditions	*Rate (percentage of control)*[a]
Complete NADPH-generating system under N_2 (Control)	100
Omit glucose-6-phosphate dehydrogenase	4 ± 1
Heat-denatured microsomes	0 ± 1
Plus NADPH (2mM)	95 ± 3
Plus NADH (2 mM)	28 ± 1
Complete system under CO	84 ± 3
Complete system under O_2 (Aerobic)	0 ± 1

[a]Rate determined as nmol of *p*-nitroaniline/30 min per mg of protein.

reminiscent of the properties of an azoreductase [95], suggesting that an enzyme of this nature may have been responsible. While preliminary, these studies also indicate that in an enzyme-catalysed process, aromatic azides are more susceptible to metabolism than alkyl azides, and that the rate of aryl azide reduction is enhanced by electron-withdrawing substituents. These observations were consistent with the previously described results for the azido group of azidomorphine (72) which, like phenethyl azide (88), proved highly resistant to bioreduction, and the azidosulphonamides (79a)–(82a), for which the electron-withdrawing 4-sulphonamido substituents would be predicted to enhance azide reduction.

Although the development of the antiretroviral nucleoside 3′-azido-3′-deoxythymidine (AZT, zidovudine) (2) will be discussed later (p. 180), an overview of studies regarding the metabolic fate of the azido group of AZT is appropriate in this section. In humans, AZT is metabolized principally to the inactive and innocuous 5′-O-glucuronide (89) with 60–80% of an administered dose being detectable in the urine as this conjugate [96]. However, Sommadossi and coworkers at the University of Alabama have obtained compelling evidence for an additional metabolic pathway involving reduction of the 3′-azido substituent. On incubation of [^{3}H]-AZT with rat hepatocyte preparations the expected 5-*O*-glucuronide was observed as the major metabolite, but significant quantities of two other compounds were also detected and characterized by mass spectrometry as 3′-amino-3′-deoxythymidine (AMT) (90) and its corresponding 5-*O*-



(2) R^1 = Me R^2 = N_3 R^3 = H

(89) R^1 = Me R^2 = N_3 R^3 = Glu

(90) R^1 = Me R^2 = NH_2 R^3 = H

(91) R^1 = Me R^2 = NH_2 R^3 = Glu

(92) R^1 = H R^2 = N_3 R^3 = H

(93) R^1 = H R^2 = NH_2 R^3 = H

(94) R^1 = H R^2 = N_3 R^3 = Glu

(95) R^1 = H R^2 = NH_2 R^3 = Glu

Glu = D-Glucuronyl

glucuronide (91) [97]. Studies with human and rat microsomal preparations indicated that azide reduction was NADPH-dependent, and also that AMT was not a substrate for the glucuronidation enzyme, each of the reduced metabolites being generated exclusively by bioreduction of the azido group of AZT or its glucuronide (*Figure 3.4*). Very recently, the same authors have verified that reduction of AZT by human hepatic microsomal preparations is indeed enzyme-mediated [98]. In the presence of NADPH, and under anaerobic conditions, the formation of AMT was diminished by various inhibitors of cytochrome P450 and metabolic activity was inducible by phenobarbitol. In addition, pre-incubation of microsomes with polyclonal antibodies to rat NADPH-cytochrome C reductase and a cytochrome P450 (IIB1) resulted in 50% and 80% inhibition of amine formation, respectively, demonstrating that these enzymes were responsible for the reaction.

Identification of AMT as an AZT metabolite was of particular interest in the light of earlier reports demonstrating the inhibitory activity of this aminonucleoside against mammalian DNA polymerase α [99] and cytotoxicity to murine tumour cell lines [100], but poor antiviral activity [100]. In clonogenic assays employing normal haematopoietic myeloid and erythroid progenitor cells isolated from human bone marrow, AMT was found to be 5- to 7-fold more cytotoxic than the parent azide [97], and although only observed at low concentrations, the possible involvement of the amine catabolite in the clinical toxicity of the drug was apparent. Investigations to elucidate the mechanism(s) underlying the toxicity of AZT towards

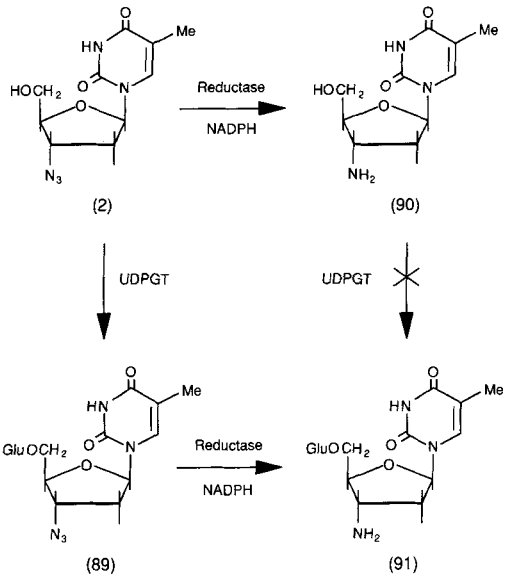
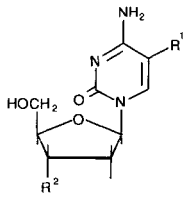


Figure 3.4. The metabolism of AZT (2).

haemopoietic cells have shown that AZT inhibits both haemoglobin synthesis and globin gene transcription in butyric acid-induced K-562 leukaemia cells *in vitro* [101]. In contrast, 2′,3′-dideoxycytidine (96) and 2′,3′-dideoxyinosine (101), had no effect on haemoglobin synthesis; this is consistent with the low incidence of anaemia associated with these agents in the clinic [102]. Analogous studies with AMT demonstrated that the metabolite was approximately equipotent to AZT as an inhibitor of globin gene transcription and steady-state mRNA levels, but inhibited haemoglobin biosynthesis to a lesser degree than the parent azide, and whereas AZT reduced haem by approximately 20% after 4 days, the same concentration of AMT was without effect on haem synthesis [103]. Not unexpectedly, AMT was more cytotoxic than AZT against K-562 cells, presumably reflecting the relative potencies of the nucleosides as inhibitors of DNA-polymerase.

The *in vitro* toxicity of AMT to mammalian cells prompted further studies to ascertain the extent to which this metabolite is formed from AZT *in vivo*. Detectable levels of AMT had been previously observed in rat faeces and ascribed to reduction of the 3′-azido group of AZT by rat intestinal microflora rather than by systemic metabolism [104]. However, the



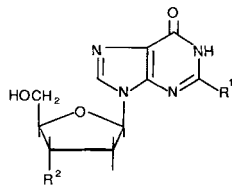
(96) $R^1 = H$ $R^2 = H$

(97) $R^1 = H$ $R^2 = N_3$

(98) $R^1 = Me$ $R^2 = N_3$

(99) $R^1 = H$ $R^2 = NH_2$

(100) $R^1 = Me$ $R^2 = NH_2$



(101) $R^1 = H$ $R^2 = H$

(102) $R^1 = NH_2$ $R^2 = N_3$

(103) $R^1 = NH_2$ $R^2 = NH_2$

formation of AMT and its glucuronide was subsequently demonstrated in the plasma and urine of rhesus monkeys following the subcutaneous administration of AZT, with plasma concentrations of the amine being sufficient to suggest that this metabolite could certainly contribute to the bone-marrow toxicity observed for AZT [105].

Pharmacokinetic and metabolism studies conducted with patients receiving AZT for the treatment of gastrointestinal malignancies enabled the Alabama team to evaluate the formation of 3′-amino-3′-deoxythymidine in human subjects [106]. Analysis of plasma, urine and bile by h.p.l.c, following intravenous infusion (1 h) of AZT containing a tracer quantity of [5-^{3}H]-AZT, confirmed the presence of AMT in serum and urine but not in bile, whereas the 5′-*O*-glucuronide (91) was observed in bile but undetectable in plasma or urine. Crucially, substantial plasma levels of AMT were observed in the study, with area under the curve values for this metabolite accounting for approximately 10–30% of the area under the curve values for unchanged drug [103, 106]. Moreover, the authors note that metabolic reduction of AZT might be even more pronounced after oral dosing as a consequence of first-pass metabolism of the drug. The amine was also found to exhibit a protracted plasma elimination half-life (2.70 ± 0.7 h) compared with that of the parent azide (1.20 ± 0.3 h), implying that accumulation of the metabolite during treatment with AZT could contribute to the clinical toxicity of the drug. Interestingly, the 5′-*O*-glucuronide conjugate (91) was found to be non-toxic to mammalian cells and inactive as an antiretroviral agent [106]. However, the *in vitro* results suggest that direct conversion of AMT is unlikely to represent a detoxification pathway for this compound as the metabolite is not a substrate for uridine 5′-diphosphoglucuronosyltransferase (UDPGT) [97].

Enzymatic reduction of the 3′-azido substituent appears to be a general biotransformation pathway for azido-substituted 2′,3′-dideoxynucleosides. The metabolism of 3′-azido-2′,3′-dideoxyuridine (AzddU) (92), an anti-HIV pyrimidine nucleoside which reportedly exhibits less myelosuppressive activity than AZT [107], was evaluated *in vitro* [108]. Utilizing the model system developed previously for AZT [97], [5-^{3}H]AzddU was incubated with isolated rat hepatocytes or microsomes and metabolites were analyzed by h.p.l.c in comparison with authentic samples of the expected metabolites. Analogous studies were also conducted with the related dideoxynucleosides 3′-azido-2′,3′-dideoxycytidine (AzddC) (97), 3′-azido-2′,3′-dideoxy-5-methylcytidine (AzddMeC) (98), and 3′-azido-2′,3′-dideoxyguanosine (AzddG) (102). In all cases, an NADPH-dependent enzyme mediated reduction of the 3′-azido group afforded substantial quantities of the corresponding 3′-amino metabolites (93), (99), (100) and (103), and by analogy with the metabolism of AZT, the authors argued that azide reduction is likely to represent the major metabolic pathway for these agents *in vivo*. However, for AzddU, much lower concentrations of the 5-*O*-glucuronide (94) were observed in rat microsomes than was reported previously for AZT [97], and the corresponding 3′-amino-5′-*O*-glucuronide (95) was not detected.

The growth-inhibitory effects of these azides and their amine metabolites were also evaluated in comparison with AZT and AMT *in vitro*, using normal haematopoietic myeloid (CFU-GM) and erythroid (BFU-E) progenitor cells isolated from human bone marrow (*Table 3.6*). Although

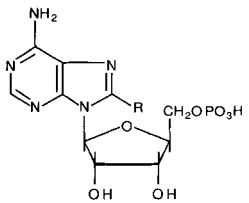
Table 3.6. COMPARATIVE CYTOTOXICITY OF 3′-AZIDO AND 3′-AMINO-2′,3′-DIDEOXYNUCLEOSIDES IN CLONOGENIC CFU-GM AND BFU-E HUMAN PROGENITOR CELLS [108]

Compound	$IC_{50}(\mu M)$	
	CFU-GM	*BFU-E*
(2)	2.0	0.6
(90)	0.4	0.1
(92)	10.0	100.0
(93)	40.0	25.0
(97)	1.0	–
(98)	40.0	100
(99)	2.0	4.0
(100)	5.0	1.0

the 3′-amino catabolites (90), (93), (99), (100) and (103) were either equitoxic or more toxic than the parent azides (2), (92), (97), (98) and (102), the 3′-amino metabolite (93) of AzddU (92) was significantly less growth-inhibitory than AMT (90), and the authors suggest that the differential toxicity of these two metabolites may account for the lack of bone-marrow toxicity observed in patients treated with AzddU [107].

Non-enzymatic bioreduction of organic azides

Under vigorous reaction conditions (100°C for several hours) azides are reduced by thiols to the corresponding amines and the possible synthetic utility of this reaction has been documented [109]. However, when conducting photolabelling experiments with nucleotide-metabolizing enzymes, Cartwright and colleagues [110] observed that this reaction could also occur under near physiological conditions if dithiols were employed as reductants. Thus, after 18 h at room temperature in the absence of light, dithiothreitol (DTT) or dithioerythritol (DTE) reduced an aqueous solution of the photoaffinity reagent 8-azidoadenosine monophosphate (104) to the 8-aminoadenosine derivative (105) in 80% yield. Reaction rates were



(104) R = N_3

(105) R = NH_2

markedly slower with the monothiol 2-mercaptoethanol ($HOCH_2CH_2SH$), much higher concentrations proving necessary to effect azide reduction, and for dithiols the reaction was two orders of magnitude faster with propane-1,3-dithiol than with ethane-1,2-dithiol (*Table 3.7*). Rates of reduction were also greatly accelerated above pH 8.0 and the authors interpreted these results in terms of a possible reaction mechanism involving initial nucleophilic attack by thiolate anion at the terminal nitrogen of the azido group, followed by intramolecular cyclization and loss of nitrogen to afford the appropriate amine and a disulphide (*Figure 3.5*).

Interestingly, the reduction of phenyl azide (1) was not observed in these

Table 3.7. RELATIVE RATES OF REDUCTION OF (104) TO (105) BY VARIOUS THIOLS (RSH) [110]

R	*Relative reduction rate*
threo-$HSCH_2CH(OH)CH(OH)CH_2$	100
erythro-$HSCH_2CH(OH)CH(OH)CH_2$	100
$HSCH_2CH_2$	2.5
$HSCH_2CH_2CH_2$	269
$HOCH_2CH(SH)CH_2$	6
$HOCH_2CH_2$	0
Ph	0
L-$H_2NCH(CO_2H)CH_2$	2

studies and this contrasts with results obtained subsequently under nearly identical conditions by Staros *et al.* at Harvard [111], who determined reduction rates for a series of aryl azides including (1) by uv/visible spectroscopy. They found that phenyl azide was rapidly reduced to aniline, albeit at a rate estimated to be some twenty-five-fold slower than that reported for the reduction of (104), and that electron-withdrawing groups on the aromatic ring increased reduction rates. Dithiols which formed five or six-membered rings upon oxidation were again found to be the most effective reductants and in accordance with the earlier studies, the reaction rate was highly pH-dependent, passing through a maximum at pH 10. However, rates of reduction were still appreciable in the physiological pH range with *N*-(4-azido-2-nitrophenyl)ethylenediamine (106) (2 mM) undergoing conversion to the arylamine (107) by DTT (10 mM) at pH 8, with a half-life of 5–10 minutes at 30°C, and the authors caution against the

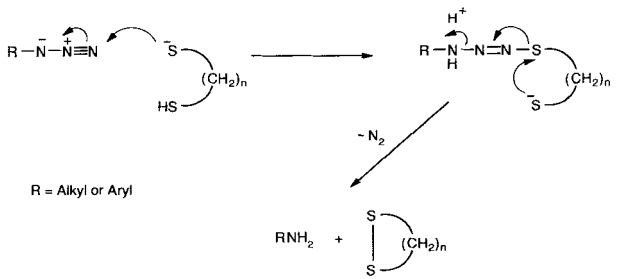
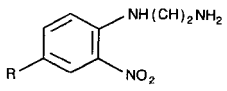


Figure 3.5. Possible mechanism for the reduction of azides by dithiols.



(106) R = N_3

(107) R = NH_2

inclusion of DTT in biological buffers employed in photoaffinity studies with azides. The same researchers subsequently reported that propane-1,3-dithiol also rapidly reduced alkyl and aryl azides under mild, but non-physiological reaction conditions in the presence of triethylamine, and confirmed that for aryl azides reaction rates were highly dependent upon substituents, with 4-nitrophenyl azide (86a) undergoing quantitative conversion to 4-nitroaniline (86b) in approximately 60 s at room temperature [112].

For aryl azides, the relative rates of metabolic or thiol-mediated reduction are clearly influenced by substituents on the aromatic ring, and we investigated this phenomenon in more detail, with a view to establishing a predictive relationship between reduction rates and substituent effects, for a series of simple substituted phenyl azides under optimized standard conditions [113]. The kinetics of azide reduction were determined by incubating stirred ethanolic solutions of the appropriate azide with DTT and triethylamine at relative final molar concentrations of approximately 1 : 100 : 230, respectively at 37°C. Samples, taken at appropriate time intervals, were analyzed by h.p.l.c. employing 1,2,4-trichlorobenzene as internal standard and pseudo first-order rate constants were calculated by following the decrease in peak height ratio using a first-order kinetic model. Under these reaction conditions the rate of reduction of 4-nitrophenyl azide (86a) was too rapid to be monitored by h.p.l.c., but was amenable to uv spectrophotometric analysis since the absorption maximum was found to be sufficiently separated from that of the product arylamine (86b).

Rates of reduction for the series of aryl azides studied are summarized in *Table 3.8*. It is evident that, for 3- and 4-substituents, the expected pattern of reactivity was observed, with electron-withdrawing substituents (Br, Cl and NO_2) resulting in a much faster rate of reduction than for unsubstituted phenyl azide, which was in turn reduced more rapidly than those aryl azides bearing an electron-donating methoxy substituent. In order to obtain some quantitative measure of the contribution of these substituents to reaction rates, the results were modelled using the Hammett relationship [114], and the linear nature of the plot of log k against Hammett constant (σ) (*Figure*

Table 3.8. EFFECTS OF VARYING SUBSTITUENT ON THE RATE OF REDUCTION OF PHENYL AZIDES ($RC_6H_4N_3$) BY DITHIOTHREITOL [113]

R	*k* (min^{-1})	*log k*	$t_{1/2}$ (*min*)	σ^a
H	0.01463	−1.8348	47.4	0
2-Cl	0.01274	−1.8948	55.4	−0.014[b]
3-Cl	0.05317	−1.2744	13.0	0.373
4-Cl	0.04149	−1.3821	16.7	0.227
2-MeO	0.001349	−2.87	513.8	−0.453[b]
3-MeO	0.008832	−2.0539	78.5	0.115
4-MeO	0.007616	−2.1183	90.2	−0.268
4-Br	0.04911	−1.3088	14.1	0.232
4-NO_2	1.4391	0.1581	0.48	0.78
2-F	0.0142	−1.8477	48.8	0.008[b]

[a][114, 115]; [b]Calculated values.

3.6) demonstrates that reduction rates are strongly governed by electronic effects. A positive value of 2.22 for the reaction constant ρ, derived from the slope ($r = 0.93$) and dependent upon the reaction and the nature of the group undergoing the reaction, indicates that azide reduction rates increase with the electron-withdrawing nature of the aromatic substituent. Although true Hammett constants cannot be derived for 2-substituents due to variable steric and conjugative effects, apparent σ_{ortho} values are valid under identical reaction conditions [115]. Values of -0.014 and -0.453 calculated for the 2-chloro and 2-methoxy groups, respectively, are thus consistent with the slower reaction rates observed for 2-chloro- and 2-methoxy-phenyl azide compared with the parent phenyl azide, and imply that steric hindrance in the region of the azido group also influences the rate of azide reduction. The implications of these results in the design of azido-substituted antifolates are discussed elsewhere.

Interestingly, our previously described studies concerning the *in vitro* biotransformation of aryl azides in murine microsomes [93] generally parallel the above results observed for the chemical reduction of azides by thiols, with electron-deficient aryl azides also undergoing metabolic reduction much more rapidly than their electron-rich counterparts or alkyl azides. These similarities have led us to speculate that a possible mechanism for the enzyme-catalyzed reduction of aryl azides may entail analogous initial attack by a sulphydryl group at the active site of the appropriate enzyme, although this hypothesis remains to be confirmed.

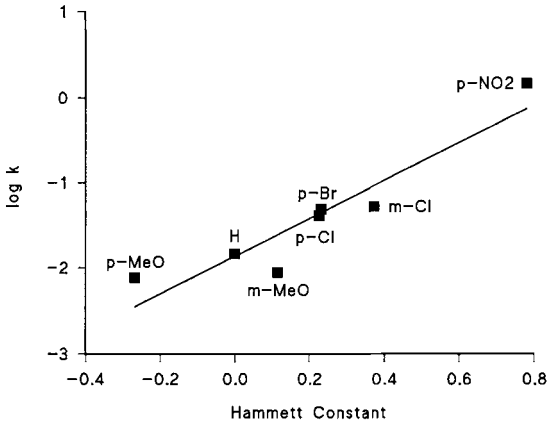


Figure 3.6. Hammett plot for the reduction of substituted phenyl azides ($RC_6H_4N_3$) by dithiothreitol. Data were taken from Table 3.8.

The Harvard group also demonstrated that the common endogenous monothiol glutathione (γ-L-glutamyl-1-cysteinylglycine) reduced aryl azides to their corresponding arylamines, albeit at a greatly reduced rate compared with DTT [111], suggesting a possible mechanism for the *in vivo* non-enzymatic bioreduction of azides to amines by GSH or other endogenous thiols. The initial antiviral evaluation of zidovudine (2) by Mitsuya *et al.* [116] was conducted over 10 days in a medium containing a 50-fold molar excess of 2-mercaptoethanol as antioxidant. Handlon and Oppenheimer investigated the propensity of this and other common thiols to reduce the azide (2) to the corresponding aminonucleoside AMT (90) [117]. The kinetics of reduction of (2) by varying concentrations of DTT, 2-mercaptoethanol and GSH were determined in degassed aqueous phosphate buffer (pH 7.2) under anaerobic conditions at 37°C, sample analysis being performed at the appropriate time intervals by reversed-phase h.p.l.c. Quantitative reduction of AZT to AMT by all three of the thiols was observed, in reactions which were first order in both thiol and azide with overall second order rate-constants of 2.77×10^{-3}, 6.55×10^{-5} and 6.35×10^{-6} M^{-1} sec^{-1} being determined for DTT, GSH and 2-mercaptoethanol, respectively. Thus, the reduction of this alkyl azide by thiols was surprisingly facile and, as expected from the previous studies [110, 111], azide reduction was most rapid for DTT, with a 5-fold molar excess of the thiol effecting complete conversion to AMT in less than an hour.

However, the observation that rates of reduction by GSH were some

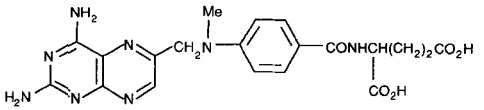
10-fold that for 2-mercaptoethanol are not in keeping with those documented for aryl azides by Staros *et al.* [111], who found only a doubling of the reduction rate. This perhaps reflects differences in experimental methodology and analysis or, alternatively, that alkyl azides are reduced to alkylamines by a different mechanism from that proposed for aryl azides above. From these results, the authors construe that azide reduction by endogenous GSH *in vivo* is plausible and speculate that AZT might function as a lipophilic AMT prodrug, undergoing phosphorylation and subsequent intracellular bioreduction to AMT triphosphate which, as such, is of comparable activity to AZT triphosphate and less inhibitory against host DNA polymerase [118].

MEDICINAL AZIDES

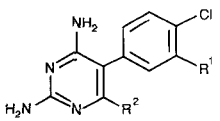
ANTIPROLIFERATIVE AZIDES

Azido-substituted antifolates

Antimetabolite drugs which act by inhibiting folate-metabolizing enzymes have a long established role in the treatment of infectious and proliferative disease [119–121]. However, folate antagonists have recently enjoyed a resurgence of interest both as cancer chemotherapeutic agents [122–124], and in the treatment of opportunist infections secondary to AIDS [125, 126]. The clinical antitumour efficacy of methotrexate (MTX) (108), a folate-analogue dihydrofolate reductase (DHFR) inhibitor, has been overshad-



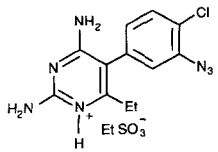
(108)



(109) R^1 = Cl R^2 = Me

(110) R^1 = Cl R^2 = Et

(111) R^1 = N_3 R^2 = Et



(112)

owed by problems of tumour resistance, and a number of lipophilic 2,4-diaminopyrimidines including metoprine (DDMP) (109) and etoprine (110), have been investigated as alternative therapeutic agents [127]. These drugs gain ingress to cells by passive diffusion processes, and hence exhibit antitumour activity against certain MTX-resistant tumours where resistance is mediated by modification of the active-transport process by which MTX enters cells [128]. However, further clinical development of DDMP was thwarted by toxicity problems arising both from the protracted biological half-life ($t_{1/2}$) of this highly lipid-soluble antifolate (plasma $t_{1/2}$ 216 h) [129], and non-folate iatrogenicity associated with inhibition of histamine-N-methyl transferase (HMT) [130].

As discussed previously, azides and halides share similar electronic and lipophilicity characteristics but differ in that the azido group may undergo biotransformation to the corresponding polar amine. Incorporation of an azido pharmacophore thus offers the opportunity of modulating the biological and pharmacokinetic properties of a drug by providing a metabolic 'handle' for inactivation on the molecule, the so-called inactive metabolite approach [131, 132]. We decided to exploit this concept in the development of novel biodegradable lipophilic DHFR inhibitors designed to combine the desirable transport properties of DDMP (109) with a $t_{1/2}$ comparable to that of MTX (108) (10 ± 2h in man) [133], in order to abrogate the cumulative toxicity associated with the former compound [129]. Our approach (*Figure 3.7*) was based on a knowledge of structure–activity relationships for diaminopyrimidine antifolates and in particular the requirement of a lipophilic substituent at the 5-position, as exemplified by

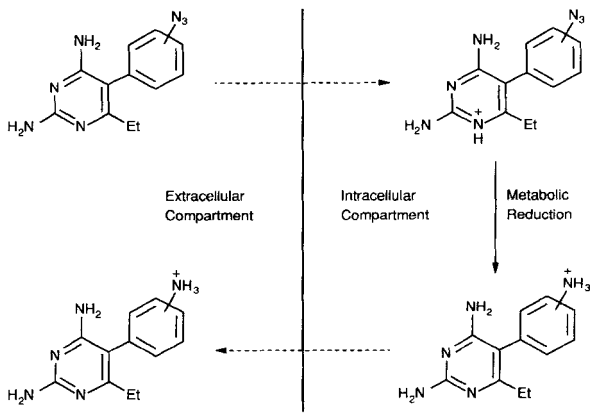
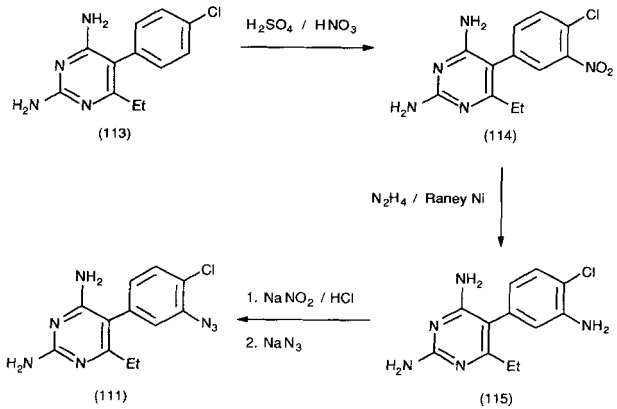


Figure 3.7. Modulation of antifolate activity as a consequence of azide bioreduction.

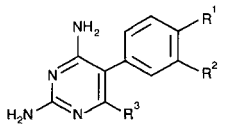
the 3,4-dichlorophenyl group of compounds (109) and (110), known to interact with a hydrophobic pocket within the active site of the enzyme [134]. Thus, incorporation of a lipophilic azido substituent into this region of the diaminopyrimidine should not compromise DHFR-inhibitory activity, or significantly alter cellular transport characteristics. However, subsequent bioconversion to the corresponding amine will introduce a polar group into a position known to favour a lipophilic substituent, and this, combined with a possible transfer of protonation from *N*-1 to the newly generated arylamine, will afford a metabolite devoid of biological activity and exhibiting physico-chemical properties favouring its rapid elimination. The diaminopyrimidine 2,4-diamino-5-(3-azido-4-chlorophenyl)-6-ethylpyrimidine (*m*-azidopyrimethamine) (MZP) (111) was the most promising of a series of azido-substituted diaminopyrimidine antifolates designed to test this hypothesis.

The synthesis of MZP is shown in *Scheme 3.1.* Nitration of the readily available antimalarial diaminopyrimidine pyrimethamine (PYM) (113) yielded *m*-nitropyrimethamine (MNP) (114) exclusively, and reduction to the corresponding amine *m*-aminopyrimethamine (MAP) (115), followed by diazotisation and azidation afforded the azide (111) in excellent overall yield [135, 136]. The extremely poor aqueous solubility of MZP (22 ± 4 μg/ml at 20°C) necessitated formulation as a water-soluble salt for biological evaluation, and *m*-azidopyrimethamine ethanesulphonate MZPES (112), with an aqueous solubility of 17.60 ± 0.45 mg/ml at 20°C, was the most appropriate for this purpose. A series of derivatives (116)–(123), differing



Scheme 3.1. Synthesis of m-azidopyrimethamine (MZP) (111).

from the parent compound with respect to the 6-substituent (Me or Et) or the aromatic substitution pattern, was prepared in a similar manner from the corresponding 2,4-diamino-6-alkyl-5-phenylpyrimidines.



(116) R^1 = Cl R^2 = N_3 R^3 = Me

(117) R^1 = N_3 R^2 = Cl R^3 = Me

(118) R^1 = N_3 R^2 = Cl R^3 = Et

(119) R^1 = OMe R^2 = N_3 R^3 = Et

(120) R^1 = OEt R^2 = N_3 R^3 = Et

(121) R^1 = NMe_2 R^2 = N_3 R^3 = Et

(122) R^1 = F R^2 = N_3 R^3 = Et

(123) R^1 = Cl R^2 = NHAc R^3 = Et

Protonation on N-1 at physiological pH is a prerequisite for the binding of diaminopyrimidines to DHFR [137] and, consequently, a pK_a of approximately 7.4 is optimal since it favours a balance between the neutral (transportable) and protonated (bioactive) forms of the molecule. ^1H and ^{13}C n.m.r. studies conducted with MZP, MNP and MAP demonstrated that protonation does indeed occur on N-1 in this series [138]. Substituents in the phenyl residue should not markedly influence pKa because the achievement of coplanarity and hence conjugation between the two rings is disfavoured on steric grounds by the 4-amino and 6-alkyl groups [139]. Indeed, molecular modelling studies conducted in our laboratory with MZP and compounds (113)–(115), utilizing *E. coli* DHFR, demonstrate that the most favourable equilibrium conformation of the bound inhibitors is achieved with the pyrimidine and phenyl rings disposed almost orthogonally [140]. These studies also showed that for MZP (111), the most favourable binding energy was observed for a conformation that oriented the azido substituent within a hydrophobic pocket in the active site cleft of the enzyme, as predicted. The physico-chemical properties of representative compounds in this series are shown in *Table 3.9*. MNP (114) and MAP (115) are the weakest and strongest bases, respectively, with PYM (113) and the azido-substituted compounds MZP and its 4-azido isomer (118) each

Table 3.9. PHYSICO-CHEMICAL PROPERTIES OF 2,4-DIAMINO-5-ARYL-6-ETHYLPYRIMIDINES [91, 135, 136]

Compound	*pK_a*[a]	*log P*[b]	*m.p. (° C)*	*Aqueous solubility (mg/ml at 20° C)*
(111) (MZP)	7.19 ± 0.10[c]	2.81 ± 0.03	197–198	0.022 ± 0.004
(113) (PYM)	7.30 ± 0.16	2.51 ± 0.03	233–234	0.029 ± 0.003
(114) (MNP)	6.84 ± 0.09	2.24 ± 0.02	203–205	0.031 ± 0.008
(115) (MAP)	7.48 ± 0.08	1.77 ± 0.04	215–217	0.784 ± 0.005
(118) (*iso*-MZP)	7.11 ± 0.11	2.79 ± 0.01	186–187	–

[a]Determined spectrophotometrically;
[b]Determined at pH 7.4 in octanol-phosphate buffer (5 mM);
[c]95% confidence limits.

exhibiting intermediate basicity, clearly reflecting the transmission of some electronic effects through the conjugated system, albeit only weakly. Not unexpectedly, the lipid solubility (log P) of the azidopyrimidines exceeded that of PYM (113), and compared favourably with the reported value of 2.82 for metoprine (109) [129], again serving to illustrate the similar lipophilic contributions made by azido- and chloro-phenyl substituents. MAP (115), the putative inactive metabolite of MZP, is an order of magnitude more polar and approximately 35-fold more water-soluble (0.78 ± 0.005 mg/ml at 20°C) than the parent azide, consistent with reduced activity against DHFR and facile elimination.

Rates of chemical, and presumably biological, reduction of the 3-azido group of azidopyrimidines will be influenced by electronic and steric effects arising from the adjacent 4-substituent. In order to estimate these effects the rates of reduction of MZP, and analogues bearing MeO, EtO, F and Me_2N in the 4-position, were studied utilizing the model DTT-triethylamine reducing system detailed above [113]. As expected, under aqueous conditions (pH 7.4, 37°C) the electron-withdrawing and relatively small ortho-halo substituents in (111) and (122) imposed fast reduction rates in this system (*Table 3.10*) while the bulkier and electron-donating methoxy- and ethoxy-substituted derivatives (119) and (120) exhibited slower rates of reduction. A similar effect was also observed for (121) where a dimethylamino group ortho to the azido substituent significantly lowered the rate of azide reduction, in keeping with the electron-donating character and greater steric bulk of the group. These results suggested that of the available

Table 3.10. KINETIC DATA FOR THE REDUCTION OF AZIDO-SUBSTITUTED 2,4-DIAMINOPYRIMIDINES BY DITHIOTHREITOL [113]

Compound	*R*	*k (min^{-1})*	*$t_{1/2}$ (min)*
111	Cl	0.0296	23.7
122	F	0.0169	41.0
119	OMe	0.0037	187.3
120	OEt	0.0034	203.8
121	NMe_2	0.0010	693.0

azido-substituted diaminopyrimidines, the 4-chloro derivative MZP would be predicted to undergo the most rapid metabolic inactivation *in vivo*, assuming that azide reduction represents the major biotransformation pathway.

Enzyme inhibitory activities, against rat liver DHFR, for ethanesulphonate salts of the azidopyrimidines are shown in *Table 3.11*. In general, the azides exhibited comparable activity to PYM (113) but were approximately 10-fold less active than the prototype diaminopyrimidine DDMP (109). The 4′-position on the phenyl ring was surprisingly tolerant to modification, methoxy- and ethoxy-pyrimidines (119) and (120) proving equipotent with the corresponding 4′-chlorophenyl derivative (112), although the introduction of a dimethylamino group (121) resulted in a modest attenuation of inhibitory activity. Limited structure–activity correlations possible within the series suggest that ethyl in (112) and (118) is preferred to methyl (116) and (117) at C-6, while 4′-azido-3′-chlorophenylpyrimidines (117) and (118) exhibit greater inhibitory activity than their isomeric 3′-azido-4′-chlorophenyl counterparts (112) and (116). The 4-fold superior potency of compound (118) over MZP was also reflected in the relative cytotoxicity of these antifolates against murine L1210 cells *in vitro*, with IC_{50} values of 8.0 nM and 70 nM being determined for (118) and (112), respectively [91]. MZP has a K_i of approximately 2.4 nM against DHFR isolated from murine L1210 leukaemia cells [92]. Replacement of the 3′-azido substituent of MZP by an amino group as for *m*-aminopyrimethamine (115), diminished inhibitory activity by approximately 50-fold,

Table 3.11. INHIBITORY ACTIVITY OF 2,4-DIAMINO-5-(AZIDOARYL)-6-ETHYLPYRIMIDINES AGAINST PARTIALLY PURIFIED RAT LIVER DHFR [91, 136]

Compound[a]	*IC_{50} (μM)*	*Inhibition constant (K_i) (nM)*
(108)[b] (MTX)	0.002	0.005 ± 0.001[c]
(109) (DDMP)	0.1	0.12 ± 0.04
(112) (MZPES)	1.3	1.60 ± 0.38
(113)[d] (PYM)	1.4	2.60 ± 0.31
(115) (MAP)	62.0	–
(116)	3.2	2.60 ± 0.76
(117)	1.0	0.82 ± 0.01
(118)	0.34	0.38 ± 0.12
(119)	0.66	1.72 ± 0.34
(120)	1.6	1.73 ± 0.34
(121)	1.6	3.00 ± 0.22

[a]Unless indicated, all compounds were evaluated as their ethanesulphonate salts;
[b]Disodium salt;
[c]95% confidence limits;
[d]Hydrochloride salt.

consistent with the introduction of a polar functionality into a formerly hydrophobic region of the molecule, and corroborating a role for this putative metabolite of MZP in the abolition of biological activity. Interestingly, the N-acetyl derivative (123), a possible conjugative metabolite of MAP, was virtually inactive as an inhibitor of DHFR.

The favourable properties and synthetic accessibility of MZP resulted in the selection of this antifolate for further biological evaluation. Antitumour activity was determined *in vivo* in a panel of murine tumours, in accordance with National Cancer Institute (NCI) protocols [141, 142], and the results are summarized in *Table 3.12*. Significant activity was observed against the P388 lymphoma, L1210 leukaemia and B 16 melanoma models, and the latter result is especially noteworthy since the tumour is refractory to DDMP (109), notwithstanding the greater potency of this agent against rat liver DHFR. Probably the most important result is that obtained for the M5076 reticulum cell sarcoma, a tumour intrinsically resistant to MTX but highly responsive to DDMP [143]. This tumour also proved to be the most sensitive to MZP, substantiating a possible therapeutic role for the antifolate in the treatment of MTX-resistant malignancies. Unpublished

Table 3.12. ANTITUMOUR ACTIVITY OF MZP (111) AGAINST SELECTED MURINE TUMOURS *IN VIVO* [91]

Tumour	*Optimum T/C (%)*	*NCI Assessment*[a]
P388 lymphoma	151	+
L1210 leukaemia	158	++
B16 melanoma	157	++
M5076 reticulosarcoma	174	++
Lewis lung carcinoma	<140	–
$CD8F_1$ mammary	24	–
Colon 38	42	–

[a]Full details of protocols are described [141, 142].

data from preliminary *in vitro* studies demonstrating collateral sensitivity to MZP in MTX-resistant murine lymphoblastoid L5178Y cells, where resistance is ascribed to defective transport-mediated uptake of the drug, also supports this proposition.

The *in vitro* metabolism of MZP in tissue homogenates prepared from the liver, kidney, spleen, intestine and heart of mice, was investigated with a view to establishing whether azide bioreduction occurred [144]. The drug was extensively and exclusively metabolized to MAP, as measured by h.p.l.c., and although some metabolic activity was detected in all of the organs studied, the liver was the major MZP-metabolizing tissue with reducing activity not being confined to a sole fraction of the liver cell. Interestingly, azide reduction by hepatic microsomal preparations was observed only under anaerobic conditions, and this activity was retained when NADH replaced NADPH but abolished by carbon monoxide, suggesting that an enzyme other than cytochrome P-450 may also be responsible. It is also of interest to compare these results with those reported from the same laboratory for the metabolism of simple phenyl azides (discussed on page 149), where reductive activity was dependent upon anaerobic conditions and an NADPH-generating system, but was unaffected by carbon monoxide [93]. Evidence that a fraction of the reducing activity was non-enzymatic was also presented. Heat-inactivated tissue homogenates retained some 10% of the activity exhibited by untreated homogenates, and the authors propose that protein thiols may be responsible for this residual reducing activity, although neither GSH nor bovine serum albumin reduced MZP under conditions identical to those employed in the incubations.

MZP was formulated for human clinical trials as the ethanesulphonate salt (MZPES) (112) at a final concentration of 10 mg/ml in nitrogen-gassed water (pH 4.1). Stability problems attributable to the azido substituent precluded sterilization of the drug by autoclaving [145], and parenteral formulations were prepared by filtration of aqueous solutions under aseptic conditions with protection from light [146]. Pre-clinical pharmacokinetic and toxicity studies in mice showed a mean elimination half-life of 4 h at 10 mg/kg and an oral bioavailability in excess of 95%. Administration of the drug as an intravenous bolus gave an LD_{10} of 18 mg/kg and an LD_{50} of 44 mg/kg, while LD_{10} and LD_{50} values of 41 mg/kg and 43 mg/kg, respectively, were observed following intraperitoneal injection. Clinical signs of toxicity in mice included reduced motor activity, muscle tremors and clonic convulsions, although no haematological toxicity or histological changes were evident. Phase I clinical studies with MZPES in patients were unusual in that the myelosuppression characteristic of antifolate drugs was not apparent at any dose following intravenous infusion, and dose-limiting toxicity, at maximum tolerated doses of 460 mg/m^2 (1 h infusion) or 800 mg/m^2 (24 h infusion), comprised of nausea, vomiting and subjective neurological disturbances [147]. The drug subsequently entered a limited phase II trial against soft tissue sarcoma at a dose of 400 mg/m^2, given by intravenous infusion over 1 h, but severe neurological toxicity coupled with the absence of any significant antitumour activity in the thirteen patients treated, resulted in the cessation of further clinical studies with this antifolate [148].

Chronic systemic administration of low-dose MTX for the treatment of severe recalcitrant psoriasis, while undoubtedly effective, carries a significant risk of hepatotoxicity [149] and attempts to circumvent this problem by topical administration have proven largely ineffective to date, due to the polar nature of the drug. MZP may offer the potential to confine antifolate activity to the proliferate layers of the skin following percutaneous absorption [150], should metabolic inactivation by cutaneous or systemic conversion to MAP occur. This possible additional therapeutic role for MZP as an alternative to systemic MTX in the treatment of psoriasis, led us to evaluate the *in vitro* growth-inhibitory properties of the antifolate against the SV-K14 keratinocyte cell-line [151]. These cells are thought to resemble the germinative cells of psoriatic lesions in that both are hyperproliferative with an impaired propensity to undergo terminal differentiation, and in the absence of an effective animal model for psoriasis, the SV-K14 cell line provides a useful primary screen for potential antipsoriatic agents [152].

In this study the inhibitory activity of MZP was evaluated, under folate-dependent and independent growth conditions, in comparison with

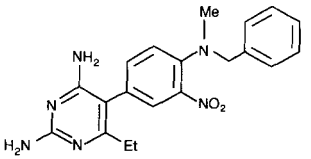
Table 3.13. GROWTH INHIBITORY ACTIVITY OF ANTIFOLATES AGAINST SV-K14 KERATINOCYTES *IN VITRO* [151]

Compound	*SV-K14 cytotoxicity ID_{50} (μM) ± SEM*		
	Folate-dependent (FD)	*Folate-independent (FI)*	*Ratio (FI/FD)*
(108) (MTX)	6.80 ± 2.3	56.7 ± 15.6	8.3
(109) (DDMP)	1.54 ± 0.3	36.6 ± 9.8	23.8
(111) (MZP)	74.6 ± 19.9	44.9 ± 6.0	0.6
(113) (PYM)	112.0 ± 22.6	60.6 ± 13.6	0.5
(115) (MAP)	73.4 ± 12.4	195.4 ± 63.5	2.7

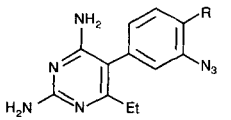
the lipophilic antifolates DDMP (109) and PYM (113), and the benchmark antipsoriatic antifolate MTX (108) (*Table 3.13*). Interestingly, although MZP proved approximately equipotent with PYM and less cytotoxic than MTX or DDMP, reflecting relative DHFR-inhibitory activities (*Table 3.11*), the significant reduction in cytotoxicity observed with the latter compounds under folate-independent growth conditions was not observed for PYM or MZP implying an additional locus of activity. PYM but not DDMP has been previously shown to exhibit clinical antipsoriatic activity following topical administration [153], and the near-identical activity profiles of MZP and PYM against SV-K14 keratinocytes suggest that analogous clinical studies with MZP may be of interest. The metabolism of MZP by SV-K14 keratinocytes and murine skin homogenate preparations was also investigated in the same study, with a view to establishing whether cutaneous transformation to MAP represented a likely mechanism for the inactivation of the drug. Following a 72 h incubation of sub-inhibitory concentrations of MZP with a monolayer of SV-K14 cells under folate-independent conditions, h.p.l.c analysis demonstrated that no metabolism of the drug to MAP had occurred, and an identical result was obtained after a 24 h incubation of MZP with murine skin homogenate in the presence of an NADPH-generating system. In both cases greater than 90% of the parent drug was recovered unchanged from the preparations following incubation, indicating that alternative possible metabolic processes were also negligible. These results imply that cutaneous biotransformation of MZP following topical administration appears unlikely, and inactivation of the drug will depend upon systemic conversion to inactive metabolites.

The interesting biological properties exhibited by MZP and related

azidopyrimidine antifolates, led us to investigate the effect of introducing an azido substituent into a structurally similar series of diaminopyrimidine antifolates developed in parallel by our group. Replacement of the 4-chloro substituent of MNP (114) by secondary or tertiary amine groups was found to afford a series of very potent DHFR inhibitors exhibiting excellent antitumour activity *in vivo* against a panel of tumours, including the M5076 reticulosarcoma, and exemplified by the *N*-methylbenzylamino analogue methylbenzoprim (MBP) (124) [154]. However, the essential role of the 3-nitro substituent in this series was evident from the profound reduction in potency observed upon replacement by an azido function in representative compounds (125)-(128), and the corresponding 3-amino or 3-unsubstituted derivatives also proved only weakly active or inactive as DHFR inhibitors.



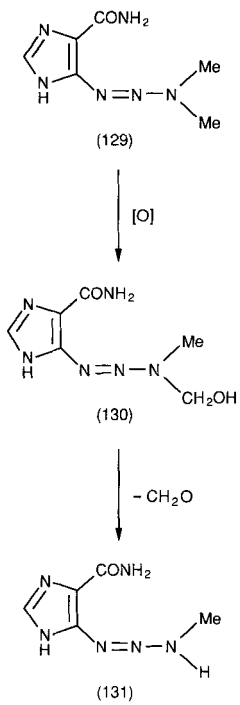
(124)



(125) R = pyrrolidin-1-yl
(126) R = piperidin-1-yl
(127) R = 4-methylpiperidin-1-yl
(128) R = 4-morpholino

Azidotriazenes

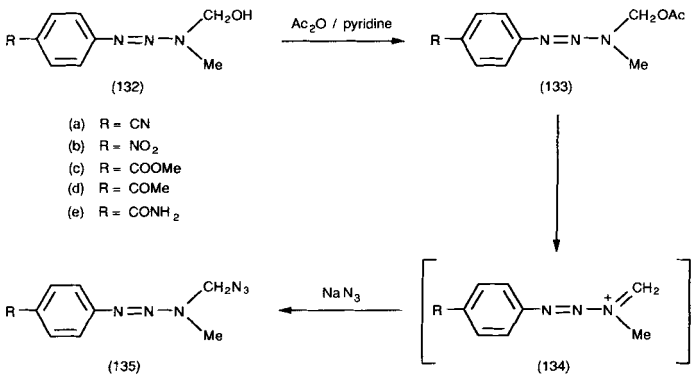
Aryl and heteroaryl dimethyltriazenes exhibit activity against a broad spectrum of murine tumours and several human tumours *in vivo*, the imidazole dimethyltriazine DTIC (dacarbazine) (129) currently being the drug of choice for the treatment of malignant melanoma [155]. Antitumour activity in this series of compounds is dependent upon metabolic oxidation of the 1-aryl-3,3-dimethyltriazine to a hydroxymethyltriazene intermediate (130) which readily loses formaldehyde affording the cytotoxic monomethyltriazene (131) (*Scheme 3.2*) [156], and one factor thought to limit the spectrum of clinical activity of DTIC is the relatively low level of metabolic activation in man compared with the mouse [157]. As part of investigations centred on the development of prodrugs of monomethyltriazines not dependent on metabolic activation, Vaughan *et al.* reported the synthesis and biological evaluation of a series of 3-azidomethyl-3-alkyl-1-aryltriazenes (135a)–(135e) [158]. These α-azidomethyltriazenes were readily synthesized from the corresponding 1-aryl-3-hydroxymethyl-3-meth-



Scheme 3.2. Metabolic activation of DTIC (dacarbazine) (129).

yltriazenes (132a)–(132e) by conversion to the 3-acetoxymethyltriazenes (133a)–(133e) and subsequent treatment with an excess of sodium azide in acetone, in a reaction thought to proceed through the iminium ion intermediates (134a–134e) (*Scheme 3.3*) [158, 159].

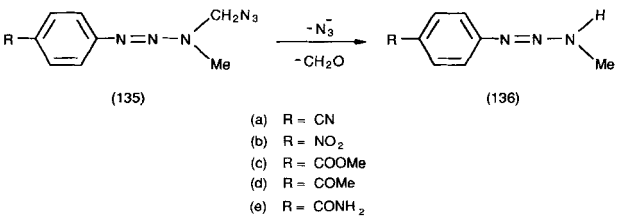
Kinetic studies conducted in phosphate buffer-DMSO showed that rates of hydrolysis of the azidomethyltriazenes (135a)–(135e) were virtually identical with those observed for the corresponding hydroxymethyl (132a)–(132e) and acetoxymethyltriazenes under the same conditions. The authors conclude that a hydrolysis pathway involving unimolecular dissociation of the azides to the iminium species (134a)–(134e) occurs, followed by rapid hydrolysis to the appropriate hydroxymethyltriazenes and loss of formaldehyde to afford the active monomethyltriazenes. Electron-withdrawing substituents on the aromatic ring retarded rates of hydrolysis of the azidomethyltriazenes in a similar manner to that observed previously for a disparate series of hydroxymethytriazenes [160], consistent



Scheme 3.3.

with the proposed hydrolytic mechanism, and suggesting that ultimate decomposition of the monomethyltriazenes (136a)–(136e) is the rate determining step in the overall hydrolysis reaction (*Scheme 3.4*).

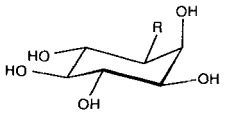
Cytotoxicity studies were conducted with compound (135c), the most readily hydrolysed of the azidomethyltriazene prodrugs prepared, against the BE and HT29 human colon carcinoma lines *in vitro*. These differ in drug sensitivity in that the BE cell line is deficient in the repair of O^6-guanine lesions and, consequently, is much more sensitive to methylating drugs than the repair competent HT29 cell line [161]. Prodrug (135c) was significantly more toxic to the BE cell line than the HT29 cell line and exhibited differential cytotoxicity similar to that reported for acetoxymethyl- and hydroxymethyl-triazenes, in keeping with the non-metabolic generation of an alkylating species assumed to be the monomethyltriazene (136c). Preliminary *in vivo* antitumour evaluation of (135c) against the murine P388 lymphoma and ADJ/PC6 plasmacytoma confirmed that the prodrug exhibited significant activity which was comparable with that for analogous triazenes [158].



Scheme 3.4.

Myo-inositol analogues

Intracellular signalling pathways are recognized as potential targets in the design of non-DNA directed antitumour agents [162]. Powis *et al.* have recently documented a remarkable example of selective antiproliferative activity with D-3-deoxy-3-azido-*myo*-inositol (142), an analogue of *myo*-inositol (137) and a putative antimetabolite of the phosphatidylinositol (PI) signalling pathway [163]. As part of long-term studies to investigate the



(137) R = OH
(138) R = H
(139) R = F
(140) R = SH
(141) R = NH_2
(142) R = N_3
(143) R = Cl

relationship between PI signal transduction, intracellular calcium release, and cellular proliferation and transformation [164, 165], several *myo*-inositol analogues were synthesized bearing modifications at the D-3 position considered to be important in signal transduction processes [162, 166, 167]. Comparative growth-inhibitory activity was determined in wild-type (WT) and oncogene transformed (OT) V-*sis*-NIH 3T3 fibroblasts *in vitro*, the latter being selected on the basis of exhibiting constitutive phosphatidylinositol-3′-kinase activity unlike the non-transformed cell line (*Table 3.14*). In order to evaluate the possibility of competition between the inhibitors and *myo*-inositol, cells were grown in *myo*-inositol-deficient culture medium and also medium supplemented with myo-inositol at a concentration (40 μM) similar to that found in human serum. No effect on the growth of either cell type was observed at this concentration of *myo*-inositol.

Deletion of the D-3 hydroxyl substituent endowed only weak activity against the v-*sis*-transformed cells and the compound (138) was inactive against the wild-type cell-line. The D-3-fluoro- and D-3-mercapto-*myo*-inositol isosteres (139) and (140) were approximately equipotent and exhibited significant, albeit relatively modest, differential activity against the wild-type and transformed NIH 3T3 cells. The D-3-deoxy-3-amino-*myo*-inositol (141) was unusual in exhibiting selectivity of growth inhibition

Table 3.14. GROWTH INHIBITION OF NIH 3T3 AND v-*sis*-TRANSFORMED NIH 3T3 CELLS BY *MYO*-INOSITOL ANALOGUES [163]

Compd.	*Growth-inhibitory activity IC_{50} (mM)*		*Ratio WT/OT*	*Inhibition of [^{3}H]-myo-inositol uptake by WT NIH 3T3 cells (K_i mM)*[a]
	Wild type NIH 3T3 cells (WT)	*v-sis transformed NIH 3T3 cells (OT)*		
(138)	NT[b]	43.2 ± 8.4	–	4.67 ± 0.19
(139)	7 ± 1.3 (48.3 ± 12.3)[c]	1.1 ± 0.6 (NT)[c]	6.4	0.63 ± 0.08
(140)	10.8 ± 3	4.0 ± 0.9	2.7	–
(141)	0.07 ± 0.01 (0.53 ± 0.07)[c]	1.1 ± 0.2 (0.59 ± 0.08)[c]	0.06	0.35 ± 0.07
(142)	51.7 ± 5.6 (18.7 ± 4.1)[c]	0.04 ± 0.01 (12.8 ± 2.1)[c]	1293	0.28 ± 0.02

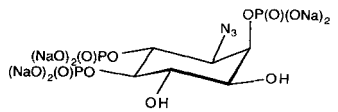
[a]K_m of [^{3}H]-*myo*-inositol = 68 ± 5 μM;
[b]Non-toxic at 33 mM;
[c]Growth inhibition in the presence of *myo*-inositol (40 μM).

for the wild-type NIH 3T3 cells, perhaps indicating a different mechanism of action from the other analogues. However, D-3-deoxy-3-azido-*myo*-inositol (142) was clearly the most potent inhibitor of the growth of v-*sis*-transformed cells and more importantly, exhibited only weak activity against the wild-type cells representing a greater than 1,200-fold selectivity in favour of the v-*sis* transformed cells. The D-3-deoxy-3-chloro-*myo*-inositol (143) reportedly also exhibited selective activity against transformed fibroblasts (data not shown) but was less potent (IC_{50} = 0.39 mM) than the azide [162]. Unfortunately, the selective antiproliferative activity of azide (142) and also the 3-fluoro analogue (139) was all but abolished in the presence of physiological concentrations of D-*myo*-inositol, and the authors conclude that although the analogues are evidently functioning as D-*myo*-inositol antagonists, they are unlikely to serve as useful antitumour agents *in vivo* unless this obstacle can be overcome.

The mechanism(s) of action of D-3-deoxy-3-azido-*myo*-inositol and related compounds remains to be determined. Although all of the D-3-substituted-*myo*-inositol analogues were competitive inhibitors of *myo*-inositol cellular uptake, this was considered unlikely to represent the principal locus of activity as the relative K_i values (*Table 3.14*) do not reflect

the large differences in growth-inhibitory activity (>700-fold) observed for the various analogues. Studies with [^{3}H]-D-3-deoxy-3-fluoro-*myo*-inositol demonstrated that this analogue is incorporated into the PI fraction of cellular lipids in both wild-type and transformed cells, and the authors speculate that *myo*-inositol analogues may function as antimetabolites by blocking intracellular PI signalling or eliciting the production of fraudulent second messengers. The precise role of phosphatidylinositol-3′-kinase in cellular proliferation and transformation is controversial, but the enzyme phosphorylates the D-3 position of the *myo*-inositol ring of PI and is thus a possible target for inhibition by D-3-substituted *myo*-inositols. However, studies with a panel of other oncogene-expressing NIH 3T3 cells, designed to demonstrate a relationship between elevated phosphatidylinositol-3′-kinase activity and inhibition of cell growth by D-3-deoxy-3-azido-*myo*-inositol or D-3-deoxy-3-fluoro-*myo*-inositol, have proven inconclusive.

Very recently, the synthesis of [^{3}H]-D-3-azido-3-deoxy-*myo*-inositol has been documented [168]. The tritiated azide will hopefully assist in unravelling the mechanism of selective antiproliferative activity and the intracellular metabolism of this and related inositol analogues. Interestingly, the same publication details an efficient synthetic route to the 3-azido-3-deoxy analogue of *myo*-inositol 2,4,5-triphosphate (144), and preliminary biological studies indicate that this azide derivative differs from 3-fluoro-*myo*-inositol 1,4,5-triphosphate (145) [169] in being notably less potent than the natural substrate in promoting the release of intracellular radiolabelled calcium ($^{45}Ca^{2+}$) from permeabilized human colon carcinoma HT29 cells.



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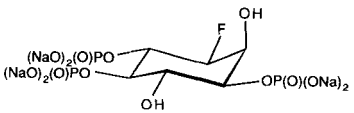
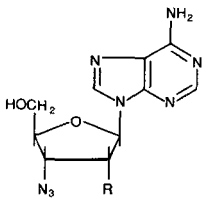


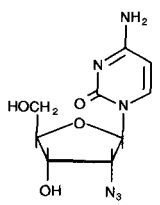
(145)

Azidonucleoside antitumour agents

Numerous azido-substituted nucleosides have been evaluated as potential antitumour agents. De Clercq *et al.* found that of a series of 2′- and 3′-azidonucleoside analogues prepared and evaluated for antitumour activity against murine L1210 cells *in vitro*, the most potent derivative was 3′-azido-2′,3′-dideoxyadenosine (146), with 3′-azido-2′,3′-dideoxyguanosine (102), 3′-azido-3′-deoxyadenosine (147) and 2′-azido-2′-deoxycytidine (148) proving moderately active [170], the activity of the last compound having been reported previously [171, 172]. AZT was also evaluated in this study



(146) R = H
(147) R = OH



(148)

and, in keeping with earlier reports [173], proved one of the least cytotoxic of the compounds studied, consistent with its role as an anti-HIV drug (see page 180). Investigations by Lin and Mancini have demonstrated that for pyrimidine nucleoside antimetabolites, a 3′-azido substituent is generally incompatible with potent cytotoxicity in mammalian cell-lines, whereas the corresponding 3′-amino derivatives often exhibit antineoplastic activity as shown in *Table 3.15* [174, 175]. For example 3′-amino-2′,3′-dideoxycytidine (99) exhibited potent cytotoxic activity against L1210 leukaemia and S-180 sarcoma cell lines *in vitro* and antitumour activity in L1210 tumour-bearing mice [171]. In contrast, the 3′-azido derivative (97) proved non-toxic to tumour cells. The differential cytotoxicity between AZT and its 3′-amino derivative AMT (90) has been discussed previously in this review (page 151).

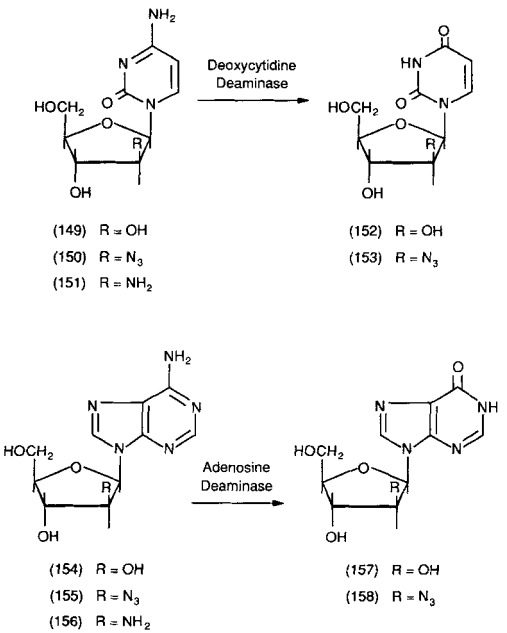
The introduction of an azido substituent on the C-2′ position of cytosine arabinoside (ara-C) (149) or adenosine arabinoside (ara-A) (154) has been found to confer favourable properties to these antimetabolites. Ara-C, one of the most effective drugs for the treatment of human acute myeloblastic leukaemia [176], is subject to rapid metabolic deamination, by deoxycytidine deaminase, to the inactive uridine derivative ara-U (152) (*Scheme 3.5*), and the drug has a half-life of approximately 12 minutes in man [177]. Efforts to circumvent this problem by modifying the 2′-*arabino*-position led

Table 3.15. GROWTH-INHIBITORY ACTIVITY OF 3′-AZIDO- AND 3′-AMINO-2′,3′-DIDEOXYNUCLEOSIDES AGAINST L1210 AND S180 CELLS *IN VITRO* [118, 174, 175]

Compound	IC_{50} *(μM)*	
	L1210 Leukaemia	*S-180 Sarcoma*
AZT (2)	inactive	inactive
(90)	1	5
(92)	inactive	inactive
(93)	18	50
(97)	inactive	inactive
(98)	inactive	inactive
(99)	0.7	4
(100)	25	20

to the synthesis of 2′-azido-2′-deoxyarabinofuranosylcytosine (cytarazid) (150) [178]. Subsequent biological studies revealed that cytarazid was highly active against several *in vitro* tumour cell lines, including L1210, human T-cell acute lymphoblastic leukaemia Molt 4F, and HeLa cells, and produced long-term survivors (>120 days) in L1210-bearing mice to which the drug had been administered [179, 180]. The nucleoside was also an effective inhibitor of the replication of HSV types 1 and 2 [180]. More recently, the *in vitro* antitumour activity of cytarazid was reported to compare favourably with ara-C against a panel of human solid tumour cell-lines, and the superior growth inhibitory activity of the azide against several human stomach adenocarcinomas was noted [181].

The 2′-azido group of cytarazid renders the nucleoside more resistant to deamination to the 2′-azidouridine derivative (153) by deoxycytidine deaminase, but was also observed to reduce substrate affinity for the deoxycytidine kinase necessary for anabolic phosphorylation to the active cytarazid 5′-triphosphate [180]. Conversely, cytarazid was a more potent inhibitor of the target DNA polymerases α and β (K_i = 0.6 and 0.7 μM, respectively) than the parent ara-C (K_i = 10 and 17 μM, respectively), and the dissimilar spectrum of antitumour activity exhibited by the two compounds was attributed to differences in stability, metabolic activation and inhibitory potency [180, 181]. Interestingly, the instability of cytarazid to thiols present in the assay media, was commented on but not pursued [180]. In view of previous discussions concerning the bioreduction of AZT



Scheme 3.5.

to AMT, the propensity of the 2′-azido substituent of cytarazid to undergo metabolic reduction to the amine 2′-amino-2′-deoxycytosine arabinoside (151), an agent that also displays potent antimetabolite activity and resistance to deoxycytidine deaminase [178], may also warrant investigation.

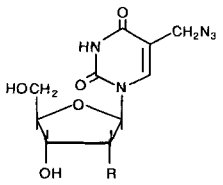
The encouraging results observed for cytarazid prompted Sandoz to conduct analogous studies with the corresponding azido analogue of adenine arabinoside (ara-A) (154), a purine antimetabolite with antiviral and antitumour activity but which is similarly rapidly inactivated by adenosine deaminase (Scheme 3.5) [182]. The susceptibility to deamination of 2′-azido-2′-deoxyarabinofuranosyladenine (arazide) (155), and its 2′-amino derivative (aramine) (156) by adenosine deaminase isolated from calf intestinal mucosa and L1210 cells, was estimated spectrophotometrically by measuring the reduction in absorbance at 265 nm. The azido-substituted nucleoside was found to be approximately 3- and 5-fold more resistant than ara-A to deamination by enzyme from L1210 cells and calf intestinal mucosa, respectively, whereas aramine was deaminated at a rate intermedi-

ate between arazide and ara-A. Preliminary *in vitro* studies conducted with L1210 cells also demonstrated that arazide was more growth inhibitory than either ara-A or aramine and, additionally, the potentiation of sub-inhibitory concentrations of all three of these agents by 2′-deoxycoformycin (pentostatin), a potent inhibitor of adenosine deaminase, was of the same rank order, with arazide proving the most active agent. Interestingly, arazide also offered a formulation advantage over the parent ara-A, being some 25-fold more water soluble [183, 184].

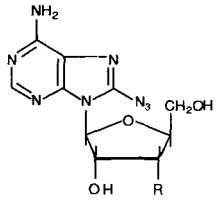
The superior antitumour activity of arazide over ara-A was subsequently established *in vivo*. Sartorelli and co-workers [185] compared the substrate affinity of the two drugs for partially purified adenosine deaminase from murine P388 lymphoma, *in vitro* cytotoxicity to P388 cells, and *in vivo* antitumour activity in P388 tumour-bearing mice, with or without co-administration of deoxycoformycin. Arazide was again a weaker substrate (K_m = 16 μM) than ara-A (K_m = 500 μM) for P388 adenosine deaminase, and the V_{max} for arazide was also 33-fold lower than that for the parent compound. The inherent cytotoxicity of arazide to P388 cells in culture was also marginally greater than that of ara-A, although no significant difference in the potentiation of growth-inhibitory activity by deoxycoformycin was observed in this study, the activity of both compounds being increased by approximately 30% on addition of the adenosine deaminase inhibitor. However, differential antitumour activity was observed *in vivo* and, whereas neither arazide nor ara-C as a single agent significantly increased the survival of tumour-bearing mice, concomitant administration of arazide (12.5–25 mg/kg) and deoxycoformycin (0.25 mg/kg) daily for 6 days resulted in the apparent complete eradication of tumour in 50% of treated animals. By contrast, a similar regimen using ara-A and deoxycoformycin did not markedly increase the survival time of tumour-bearing mice, confirming the superior antineoplastic activity of the azide and implying that pharmacological and/or pharmacokinetic differences may exist between the two adenosine antimetabolites. In common with ara-A, the antitumour activity of arazide appeared to derive from the inhibition of DNA synthesis, following conversion to the active 5′-triphosphate which directly inhibits DNA polymerase and RNR activities. Indeed, the ability of arazide, and the isomeric 2′-azido-2′-deoxyribofuranosyladenine, to form triphosphate nucleotides after incubation with erythrocytes or Sarcoma 180 cells in the presence of deoxycoformycin, has subsequently been demonstrated [186].

The deoxyuridine analogue 5-azidomethyl-2′-deoxyuridine (159) was one of a large number of compounds investigated as inhibitors of thymidylate synthase and thymidine kinase from L1210 cells, but the compound was

only weakly active [187]. Modest *in vitro* antitumour (L1210, Sarcoma 180) and antiviral activity (HSV-1), preventable by the addition of exogenous 2′-deoxypyrimidine nucleosides, has also been reported for this compound [188]. A number of other nucleoside analogues bearing azido substituents on the pyrimidine or purine moiety have also been reported to exhibit antiproliferative activity *in vitro* [189]. Compounds were evaluated both as inhibitors of RNR and for growth-inhibitory activity in murine adenocarcinoma EMT6 cells, with two analogues, 8-azidoadenosine (162) and 5-azidomethyluridine (160) proving moderately cytotoxic, although only 8-azidoadenosine exhibited significant inhibitory activity against RNR. The authors found that a 2′-hydroxyl substituent was an absolute prerequisite for biological activity with 8-azido-2′-deoxyadenosine (161) being totally inactive, while the di- or triphosphate derivatives of (162) could still serve as substrates for RNR and as such represent potentially useful photoaffinity labelling reagents for the enzyme.



(159) R = H
(160) R = OH



(161) R = H
(162) R = OH

AZIDO-SUBSTITUTED ANTI-INFECTIVE AGENTS

Zidovudine and related nucleosides

Without doubt the most celebrated example of the application of an azido substituent in drug design is 3′-azido-3′-deoxythymidine (AZT) (2), the first drug to be licensed for the treatment of AIDS (note that 'deoxy-' at the 2′-position is implicit in the name 'thymidine', making this a 2′,3′-dideoxynucleoside). Initially synthesized and evaluated as a potential antitumour agent [100, 118, 173,], AZT was selected for clinical evaluation in 1985 as one of the most promising of over 300 compounds evaluated by the NCI for *in vitro* inhibitory activity against human immunodeficiency virus (HIV), the pathogen responsible for AIDS [116, 190]. The drug was

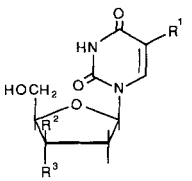
administered to patients in the same year and marketed subsequently by Burroughs Wellcome Co. as zidovudine, after multi-centre clinical trials over the following months demonstrated unequivocally that AZT was effective in the treatment of AIDS [191]. AZT gained approval for clinical use with a rapidity unprecedented in modern drug development and currently remains the first-line drug of choice for the treatment of HIV infection, although 2′,3′-dideoxycytidine (zalcitabine) (96) and 2′,3′-dideoxyinosine (didanosine) (101) have more recently also secured clinical approval from the U.S Food and Drug Administration (FDA). A computer search of the literature for AZT and related compounds, for the period from 1986–1993, generated in excess of 2000 entries including a number of comprehensive reviews documenting the development, clinical efficacy and toxicology of 2′,3′-dideoxynucleosides [191–200]. Since a detailed survey is clearly beyond the scope of this review, only those studies directly relating to the role of the azido substituent in the biological activity and toxicity of azido-substituted antiretroviral agents will be discussed.

Although the exact intracellular mechanism of action of AZT and related dideoxynucleosides remains uncertain, antiretroviral activity is undoubtedly associated both with the competitive inhibition of reverse transcriptase (RT) and termination of viral DNA chain-elongation. In common with other dideoxynucleosides, the drug has no intrinsic anti-HIV activity but requires phosphorylation by cellular kinases to afford the 5′-monophosphate, which undergoes further anabolism by host enzymes to the diphosphate and triphosphate derivatives [116, 201]. As a structural analogue of the natural substrate thymidine-5′-triphosphate, AZT-5′-triphosphate may then function as an antimetabolite by directly inhibiting viral RT or competing with the normal substrate for incorporation into proviral DNA, where it functions as a terminator of DNA chain-elongation [202].

Selective toxicity has been attributed to the greater affinity of AZT-5′-triphosphate for viral RT over host cell DNA-polymerase α, and the triphosphate is 100–300 times more potent an inhibitor of the former enzyme *in vitro* [116]. However, DNA polymerases β and γ are reportedly more sensitive [203, 204] and inhibition of these enzymes may account for at least some of the toxicity associated with AZT therapy, most notably that of myelosuppression manifesting as anaemia and neutropenia. Moreover, incorporation of dideoxynucleosides, including AZT, into host DNA has been reported [205, 206] and may correlate with host toxicity. At the mono-and tri-phosphate levels AZT also directly inhibits its own further phosphorylation, and that of thymidine, with the resultant depletion of thymidine 5′-triphosphate contributing to the antiviral activity and

untoward cytotoxic effects of the drug [201, 207]. AZT-monophosphate additionally functions as an inhibitor of the RNAse H component of HIV RT [208], but the importance of this activity in the antiviral activity of the drug is uncertain.

Broder and colleagues observed that virtually any pyrimidine or purine with a 2′,3′-dideoxyribofuranosyl moiety inhibited HIV replication *in vitro* to some extent, but that many exhibited little selectivity and were also highly toxic to mammalian cells at antiviral concentrations [190]. AZT lacks the 3′-OH substituent necessary for 5′,3′-phosphodiester formation and will thus function as a DNA chain-terminator following phosphorylation and incorporation into viral DNA. However, if the absence or substitution of a hydroxyl group at the 3′-position were the sole criterion for antiviral activity, then allowing for pharmacokinetic differences, many 2′,3′-dideoxynucleosides might be expected to exhibit comparable activity *in vitro*, including 3′-deoxythymidine (163) which, nevertheless, is a much weaker inhibitor of HIV replication (*Table 3.17*) [209]. The 3′-azido substituent of AZT imparts excellent substrate activity (K_m = 3.0 μM) for the host thymidine kinase enzymes involved in anabolic phosphorylation of the drug to the active triphosphate [201]. However, the 3′-unsubstituted analogue (163), being a much poorer substrate for phosphorylation is accordingly much less active [209], and efficient intracellular conversion to



(163) R^1 = Me R^2 = H R^3 = H

(164) R^1 = Me R^2 = N_3 R^3 = H

(165) R^1 = Et R^2 = H R^3 = N_3

(166) R^1 = I R^2 = H R^3 = N_3

(167) R^1 = Br R^2 = H R^3 = N_3

(168) R^1 = Me R^2 = H R^3 = I

(169) R^1 = Me R^2 = H R^3 = F

(170) R^1 = Me R^2 = H R^3 = CN

(171) R^1 = H R^2 = H R^3 = H

the active triphosphate appears to be a prerequisite for all 2′,3′-dideoxynucleosides exhibiting significant protective effects against HIV *in vitro* [210–212].

Following conversion to the triphosphate, the role of the azido substituent of AZT in the interaction of the drug with viral RT is also uncertain. Enzyme inhibition studies have demonstrated that HIV RT has a K_m of 2.8 μM for the natural substrate thymidine-5′-triphosphate, whereas AZT-5′-triphosphate is a potent competitive inhibitor of the enzyme (K_i = 0.04 μM), and by contrast, the triphosphate exhibits K_i values of 230 μM and 70 μM for mammalian DNA polymerases α and β, respectively [201]. The triphosphates of other clinically useful dideoxynucleosides, including those derived from 2′,3′-dideoxycytosine (96) and 3′-azido-2′,3′-dideoxyuridine, (92) are also selective and potent inhibitors of isolated RT [204, 214].

However, equivalent potency of enzyme inhibition has been reported for the triphosphates of 3′-deoxythymidine and 3′-amino-3′-deoxythymidine despite the weak antiviral activity of the parent nucleosides (163) and (90) *in vitro*, both of which are poor substrates for phosphorylation [213, 214]. These observations suggest that the antiviral activity of 2′,3′-dideoxynucleosides may depend less on the relative inhibitory activities of the triphosphates against isolated RT, but rather reflects the affinity of the parent nucleosides for the cellular kinase enzymes effecting triphosphorylation. Nevertheless, other studies of RT inhibition by nucleoside triphosphates have demonstrated a defined SAR for modifications at the 3′-position [215], and it is likely that the azido substituent of AZT contributes both to the requisite substrate activity for the cellular phosphorylating enzymes, and the specificity of RT inhibition following conversion to the triphosphate.

Some caution must be exercised when interpreting SARs among the 2′,3′-dideoxynucleosides because these agents are not equivalent with regard to activity or toxicity *in vitro* or *in vivo*, and each may utilize a separate anabolic pathway, with minor structural modifications profoundly altering affinity for a particular activating enzyme [197, 198, 216]. For a particular dideoxynucleoside, potency of antiretroviral activity also varies widely in different infected cell lines (*Table 3.16*), reflecting both the relative activities of anabolic and degrading enzymes within each host cell and the intracellular levels of competing endogenous deoxynucleoside phosphates [217]. Thus, AZT is markedly more active than 2′,3′-dideoxyinosine (101) in phytohaemagglutinin-stimulated human peripheral-blood mononuclear cells *in vitro*, but some twenty-fold less potent in non-dividing human monocyte-macrophages infected with a monocytotropic strain of HIV, and

Table 3.16. ACTIVITY OF AZT (2), DIDEOXYCYTIDINE (96) AND DIDEOXYADENOSINE (184) AGAINST HIV IN DIFFERENT CELL SYSTEMS [217]

Cell system (virus strain)	*EC_{50} (μM)*		
	(2)	*(96)*	*(184)*
MT-4 (IIIB)	0.006	0.06	10
ATH-8 (IIIB)	2.4	0.2	7
PBMC (LAV-1)	0.006	0.011	3-5
Monocytes/macrophages (HTLV-$IIIB_{a-L}$)	0.2	0.002	0.01

the latter cell line is thought to better represent the *in vivo* situation [217]. The 3′-azido substituent of AZT certainly makes an important contribution to activity and selectivity but the precise mechanism(s) by which this occurs is uncertain. The lipophilic azido group may confer favourable pharmacokinetic properties to the drug (log $P = 0.964$) which, unlike many other nucleosides, can gain cellular ingress *via* passive diffusion [218]. De Clercq *et al.* found that the introduction of an azido substituent uniformly conferred enhanced lipophilicity to a wide range of nucleosides, and that relatively minor changes in the sugar moiety influenced lipid solubility dramatically [219]. AZT also readily penetrates the blood-brain barrier with cerebrospinal fluid concentrations averaging 55% of that in plasma 3–4 h after drug administration [220].

SARs have been elucidated for a number of azido-substituted derivatives of AZT (*Table 3.17*). Epimerisation of the 3′-N_3 substituent to afford *threo*-AZT (164) completely abolished antiviral activity [197], indicating that the orientation of the azido group at the 3′-position is crucial, and that the interaction of the azido pharmacophore with its biological target arises through association with a sterically constrained domain. Chu and colleagues synthesized a series of pyrimidine nucleosides, structurally related to AZT, and evaluated these in HIV-1 infected human peripheral blood mononuclear (PBM) cells [221]. Of those AZT analogues modified at the 5-position, none proved more active than the parent compound, although the 5-ethyl analogue (165) exhibited significant antiviral activity in these studies despite reports to the contrary in studies with other cell lines [222]. Further homologation at the 5-position greatly reduced or abrogated activity. The 5-H analogue was also identified in these studies as a potent and selective anti-HIV agent, and the selection of this dideoxynucleoside

Table 3.17. ACTIVITY AND TOXICITY OF PYRIMIDINE NUCLEOSIDES AGAINST HIV-1 IN HUMAN PERIPHERAL BLOOD CELLS [221]

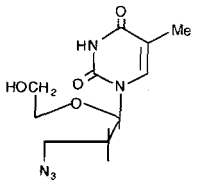
Compound	*Antiviral activity* EC_{50} (μM)[a]	*Cytotoxicity in uninfected cells* IC_{50} (μM)[b]
(2) (AZT)	0.002–0.009	200
(90)	>100	>100
(92) (AzddU)	0.18–0.46	1000
(163)	0.17	>100
(165)	0.056–1.00	1000
(166)	1.14	>100
(167)	1.04	>100
(168)	46.3	>100
(171)	96.8	ND
(174)	>100	>100
(182)	0.66–1.19	>400

[a]Median effective concentration;
[b]Median cytotoxic concentration in uninfected cells.

(AzddU) (92) for further clinical evaluation has been discussed previously. Introducing an iodo or bromo group resulted in a considerable loss of potency compared with AZT, but these derivatives still retained some antiviral activity. Indeed, very recent studies at the NCI indicate that 3′-azido-2′,3′-dideoxy-5-iodouridine (166) and 3′-azido-2′,3′-dideoxy-5-bromouridine (167) are excellent antiretroviral agents *in vitro*, combining minimal toxicity to uninfected human lymphoid H9 cells, with efficient phosphorylation, potent inhibition of RT (K_i = 0.028 and 0.043 μM, respectively) and relatively poor activity against host cell DNA polymerase α (K_i = 42.0 and 47.0 μM, respectively) [224].

The importance of a 3′-N_3 substituent of AZT has been established by its removal (163), reduction to the corresponding amine (90), or substitution by an iodo group (168), with all three modifications dramatically compromising antiviral activity [221]. However, the introduction of a 3′-fluoro group has been reported to confer activity with 3′-fluoro-3′-deoxythymidine (169) demonstrating respectable antiviral activity *in vitro*, albeit in a different cell assay [222]. The acyclic analogue of AZT (172) has also been synthesized and evaluated as an antiviral agent, but was found to be virtually inactive against HIV or HSV at concentrations of up to 100 μM [223].

Numerous other 3′-substituted dideoxynucleosides have been synthesized and evaluated [190, 197] but to date, few have exhibited comparable activity to AZT or the 3′-unsubstituted dideoxynucleosides in *in vitro* antiviral screens, and some have proven surprisingly poor inhibitors. For example, the cyano group, being a pseudohalide of low steric bulk and similar electronic character to the azido group, represented a particularly attractive target 3′-substituent, and initial reports of the potent antiviral activity of 3′-cyano-3′-deoxythymidine (cyanothymidine) (170) generated considerable media excitement [225]. However, in subsequent biological studies conducted with cyanothymidine prepared by chemists at Pfizer, who developed an unambiguous synthetic route [226], the compound was completely devoid of antiviral activity. The authors conclude that earlier reports of activity were probably due to contamination by the alkene (173), formed as a by-product in the synthesis and known to inhibit HIV replication *in vitro*. That the structurally similar cyano analogue should be inactive compared with the parent azide, suggests that a 3′-cyano substituent either renders the nucleoside a non-substrate for the phosphorylating enzymes essential for elaboration to the triphosphate, or precludes the interaction with reverse transcriptase following conversion to 3′-cyanothymidine triphosphate.



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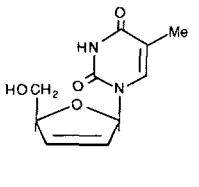

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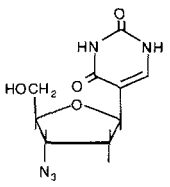
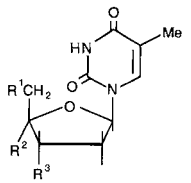


(174)

Transposing the 3′-N_3 and 5′-OH groups of AZT (175) also abolished antiviral activity [221]. Surprisingly, in a separate study [213], the 3′,5′-diazido derivative (176) was found to exhibit weak but significant



(175) $R^1 = N_3$ $R^2 = H$ $R^3 = OH$

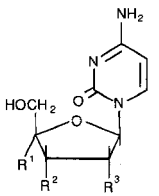
(176) $R^1 = N_3$ $R^2 = H$ $R^3 = N_3$

(177) $R^1 = OH$ $R^2 = N_3$ $R^3 = OH$

(178) $R^1 = OH$ $R^2 = N_3$ $R^3 = H$

(179) $R^1 = OH$ $R^2 = N_3$ $R^3 = OMe$

activity, implying that a 5′-OH group may not be an absolute prerequisite for substrate affinity for thymidine kinase. The alternative possibility that *in situ* chemical or enzymic formation of AZT had occurred through hydrolysis of the 5′-azidomethyl group, was regarded by the investigators as unlikely, but cannot be discounted in light of evidence for alkyl azide hydrolysis presented elsewhere in this review. The tenet that antiretroviral activity is immutably associated with the absence of a 3′-hydroxyl group has been confuted by Syntex Research, who have recently discovered potent activity among a series of 4′-azidonucleosides synthesized as part of a programme directed at agents structurally dissimilar to AZT [227]. Of the series of 4′-azido substituted pyrimidine and purine analogues evaluated for anti-HIV activity and host-cell cytotoxicity, activity resided exclusively in 4′-azido-2′-deoxy-β-D-nucleosides, with the α-L epimers being devoid of activity. A 2′-hydroxy group was also detrimental as demonstrated by the



(180) $R^1 = N_3$ $R^2 = OH$ $R^3 = OH$

(181) $R^1 = N_3$ $R^2 = OH$ $R^3 = H$

(182) $R^1 = H$ $R^2 = N_3$ $R^3 = H$

Table 3.18. INHIBITORY ACTIVITY OF 4′-AZIDONUCLEOSIDES ON THE CYTOPATHOGENICITY OF HIV (LAV[III_B]) IN A3.01 CELLS AND CELLULAR TOXICITY [227]

Compound	IC_{50} (μM)	CC_{25} (μM)[a]	CC_{100} (μM)[b]	*Selectivity index* (CC_{25}/IC_{50})
AZT (2)	0.01	825	3300	82,500
(177)	0.01	8	200	800
(178)	NA	22	200	–
(179)	20.4	>200	>200	>9.8
(180)	NA[c]	8.2	74.1	–
(181)	0.004	0.21	1.9	53
(183)	0.003	0.21	1.9	70

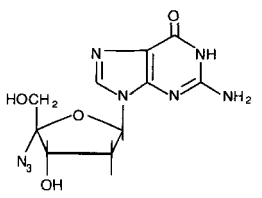
[a]Concentration of drug at which 25% of cells were destroyed;
[b]Lowest concentration at which complete destruction of cells occurred;
[c]Not active at cytotoxic concentrations.

inactivity of the riboside derivative 4′-azidocytidine (180), whereas, paradoxically, deletion or methylation of the 3′-hydroxyl substituent (178) (179) virtually abolished activity in this series (*Table 3.18*). Indeed any further modification of the 2′ or 3′ position invariably reduced activity.

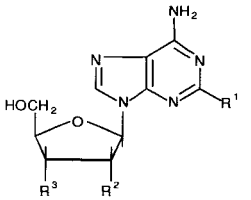
The 4′-azido analogues of 2′-deoxycytidine (181) and 2′-deoxyguanosine (183) were extremely potent inhibitors of viral replication, but exhibited poor selective toxicity compared with AZT. Optimal activity was observed for 4′-azidothymidine (177) which exhibited a similar inhibitory profile to AZT in a range of HIV-infected cells, but nevertheless was more cytotoxic in several other cell lines, probably reflecting differential sensitivity of cellular polymerases to the azidonucleoside. Perhaps more importantly, 4′-azidothymidine was equally effective *in vitro* against AZT-resistant and sensitive HIV clinical isolates in these studies, implying that the observed cross-resistance to other azido-substituted dideoxynucleosides may not extend to this structurally unique series of azidonucleosides. More comprehensive biological studies conducted with 4′-azidothymidine have shown that the nucleoside is a substrate for thymidine kinase, but that the monophosphate has a low affinity for thymidylate kinase and thymidylate synthase, indicating that activation to the triphosphate should proceed without adversely affecting cellular thymidine metabolism [228]. The mechanism of action of 4′- azidothymidine has not been fully elucidated but preliminary studies confirm that the drug can still function as a DNA

chain-terminator despite possessing a 3′-hydroxy group [227]. Moreover, it appears that, unlike conventional dideoxynucleoside antiretroviral agents, 4′-azidothymidine is internally incorporated into cellular DNA although the significance of this in terms of activity and toxicity is uncertain.

A large number of 3′-azido analogues of AZT, modified in the heterocyclic base, have been synthesized and evaluated for antiretroviral activity, and SARs have been elucidated [221, 222, 229–231] (*Table 3.17*). In the pyrimidine series, replacement of thymine by uracil does not compromise antiviral activity as demonstrated by the clinically effective AzddU (92). The significance of the 3′-azido substituent in AzddU is evident from the inactivity of 2′,3′-dideoxyuridine (171) which differs only in lacking an azido group at this position. Enzyme kinetic studies have demonstrated that the 5′-triphosphate of this dideoxynucleoside is a potent selective inhibitor of RT and also functions as a DNA chain-terminator [232]. However, phosphorylation of the 3′-unsubstituted uridine is highly inefficient and probably accounts for the feeble *in vitro* antiviral activity observed, whereas, by contrast, AzddU is efficiently phosphorylated and the 5′-triphosphate is of comparable potency to AZT-triphosphate as an inhibitor of RT [233]. The *C*-nucleoside analogue of AzddU (174) has also been prepared but failed to exhibit any significant *in vitro* anti-HIV activity in PBM cells [221]. The *in vitro* activity of the corresponding cytosine derivative of AZT appears to depend on the cell system employed. Thus, 3′-azido-2′,3′-dideoxycytidine (182) was reported as virtually inactive in the initial NCI screening programme [190], and this compound, together with its 5-methyl analogue, was also without antiviral activity in infected ATH8 T-cells [222]. These results are in contrast to those of Chu *et al.* [221] who found that the 3′-azidocytidine analogues were effective antiretroviral agents in the PBM cell line, and at least tenfold more active than was reported previously in the same cell system [230].



(183)



(184) $R^1 = H$ $R^2 = H$ $R^3 = H$

(185) $R^1 = H$ $R^2 = N_3$ $R^3 = H$

(186) $R^1 = NH_2$ $R^2 = H$ $R^3 = N_3$

Numerous purine derivatives of AZT have also been described and 3′-azido substitution in 2′,3′-dideoxyadenosine, 2′,3′-dideoxyguanosine and 2′,3′-dideoxyinosine affords compounds which are active. These appear to offer no therapeutic advantages over the corresponding 3′-unsubstituted dideoxynucleosides, and in the case of the azidoadenosine analogue (146), the azido substituent may enhance toxicity [190, 197]. Thus, De Clercq et al. [234] reported that of the *erythro* and *threo* epimers of 2′- and 3′-azido-2′,3′-dideoxyadenosine, the 3′-*erythro* derivative (146) was slightly more active than 2′,3′-dideoxyadenosine (184) in protecting MT-4 cells against the cytopathogenicity of HIV *in vitro*. In comparison with AZT, however, *erythro*-3′-azido-2′,3′-dideoxyoadenosine (146) was 800-fold less potent and markedly more cytotoxic, and whereas the *erythro* 2′-azido congener (185) exhibited the most favourable therapeutic index, this compound was 1000-fold less active than AZT. In keeping with the reported inactivity of *threo*-AZT [197], the *threo* epimers were virtually inactive in these studies. Only one azidopurine derivative, the 3′-azido analogue of 2′,3′-dideoxy-2,6-diaminopurine riboside (186), appears to offer any clinical potential and has been reported to combine good *in vitro* antiretroviral activity in the MT-4 cell line, with some degree of selective toxicity [193, 219, 235].

Additional evidence of a unifying functional role for the 3′-azido substituent in the interaction of dideoxynucleosides with RT, stems from studies with HIV strains resistant to AZT. Drug-resistant strains as much as 100-fold less sensitive to AZT have been isolated from patients receiving long-term therapy with the drug (>6 months) [236], and mutations in up to four amino acid residues of the RT gene have been identified by comparative nucleotide sequence analysis of sensitive and resistant isolates, the level of resistance correlating with the number of mutations present [237]. High level resistance appears to be associated with amino acid substitutions at positions 67, 70, 215 and 219 of the RT coding region, with mutations at position 215 (tyrosine or phenylalanine for threonine) being especially common, and these mutations are thought to result in modification of the charge or alpha helix content of the enzyme catalytic site [198].

Interestingly, AZT-resistant strains were found to be cross-resistant to related 3′-azido-substituted dideoxynucleosides including AzddU (92) and AzddG (102) but, in general, retained their sensitivity to those dideoxynucleosides without an azido group, most notably 2′,3′-dideoxycytidine (96) and 2′,3′-dideoxyinosine (101) [238–240]. This observation suggests that the 3′-unsubstituted nucleosides should be effective in the treatment of infection due to AZT-resistant strains, although the clinical implications of resistance to AZT are as yet unknown, and strains resistant to 2′,3′-dideoxycytidine

and 2′,3′-dideoxyinosine have recently been isolated from patients undergoing long-term treatment [240, 241]. Mutations within the active site of RT are constrained by the requirement for binding of the natural 2′-deoxynucleoside substrate, and it is plausible that the enzyme is modified so that association of the 3′-hydroxyl function of the substrate with a putative binding domain occurs with exclusion of the larger azido substitutuent. Such a conformational change is consistent with the observed cross-resistance to other 3′-azido dideoxynucleosides and the sensitivity of the mutant enzyme to 2′,3′-dideoxynucleosides, which would be less subject to such steric limitations. However, RT isolated from AZT-resistant HIV variants exhibits no significant difference (IC_{50}, K_m or K_i) from that of the wild-type enzyme with respect to AZT-triphosphate [197, 236, 242], implying either that inhibition studies employing isolated enzyme are artifactual or, that an anabolite other than AZT-triphosphate may be the true intracellular antiretroviral species.

Unfortunately, no crystal structure determination of AZT bound to HIV-1 RT has been reported to date, although the structure of the RNAase H domain of the enzyme has been solved [243]. More recently, a ternary complex comprising of RT, a monoclonal antibody FAB fragment and a DNA primer template has been published and the putative active site of the polymerase identified [244], suggesting that the visualization of complexes of the enzyme with nucleoside analogues is imminent. A number of X-ray analyses of the crystal structure of AZT have been reported and provide some information regarding a possible role for the 3′-azido group. Camerman and colleagues found that AZT comprises of two independent molecules in the crystal asymmetric unit, differing primarily in the glycosyl torsion angle but having very similar conformations, and these were compared with the crystal structure of thymidine 3′-monophosphate [245]. Interestingly, superimposition of the pyrimidine bases of all three molecules revealed very similar overall conformations, with the deoxyribose rings of the AZT molecules occupying spatial positions close to that of the corresponding thymidylate sugar. Thus, the azido substituent does not appear to significantly alter the conformation of the nucleoside. In addition, when superimposed, two of the azido nitrogens (N4 and N6) of the AZT molecules were observed to occupy positions near to the C3′-oxygen and a phosphate oxygen of thymidine monophosphate, and the authors suggest that the azido group may compete with these oxygens for hydrogen-bonding at the nucleotide binding sites of the enzyme as shown in *Figure 3.8*. Such an interaction could conceivably arise following incorporation of AZT into viral DNA, with the azido group preventing the chain-lengthening nucleotide from binding to the polynucleotide binding site and hence

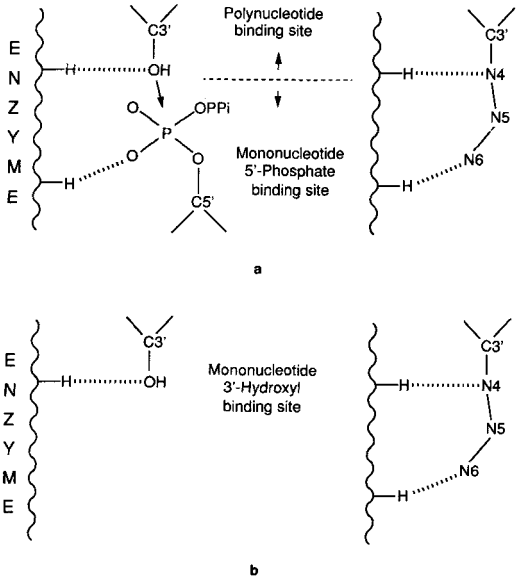


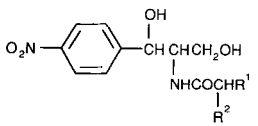
Figure 3.8. Schematic representation of the possible binding of thymidine (Left) and AZT (Right) to reverse transcriptase when incorporated into DNA (a), and competing for the enzyme thymidine-binding site (b). (Reproduced with permission from reference [245]; copyright 1987, The National Academy of Science USA).

contributing to chain termination (*Figure 3.8a*). Alternatively, if AZT simply competes with thymidylate for association with the enzyme nucleotide binding site, the additional hydrogen bond that could be formed between RT and the terminal N6 azido nitrogen might explain the stronger binding affinity of AZT as compared to the natural substrate (*Figure 3.8b*).

The existence of two independent molecules, as a hydrogen-bonded dimer, in the crystal structure of AZT has been confirmed subsequently [246]. However, in contrast to the previous report, the two molecules exhibited different conformations, although the azido group did not appear to radically alter the conformation of the molecules compared with thymidine. In this crystal structure, the azido substituents were not involved in any hydrogen bonding but served as terminators of base stacking with the azido groups forming a sandwich either side of two stacked bases. The authors propose that conformational similarities may explain why AZT is a good substrate for thymidine kinase, but that stereoelectronic rather than conformational effects are responsible for the antiviral activity of the drug.

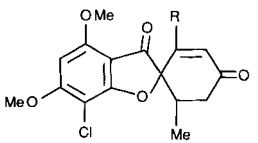
Azido-substituted antibiotics

The introduction of an azido substituent reportedly imparts favourable biological properties to a number of antibiotics and has been claimed to improve activity against Gram-negative bacteria [247]. Thus, substitution of the dichloroacetyl group ($COCHCl_2$) of chloramphenicol (187) by azidoacetyl ($COCH2N_3$) afforded azidamphenicol (leukomycin-N) (188), an antimicrobial agent developed by Bayer for topical use as an ointment and in eye drops [248, 249]. An azido derivative of the antifungal agent griseofulvin (189), where the 2′-methoxy substituent is replaced by an azido group (190), has been patented by Schering as an orally-active agent for the treatment of tinea infections of the skin, hair or nails [250, 251]. The penicillin derivative α-azidobenzylpenicillin (azidocillin) (193) evolved from studies conducted in Sweden by Astra Laboratories, as part of a programme to prepare semi-synthetic penicillins with amino substituents in the acyl side-chain, following the success of ampicillin (192) [252]. Acylation of 6-aminopenicillanic acid (6-APA) (194) with amino acids requires protection of the amino function of the latter, and the most effective method entailed prior



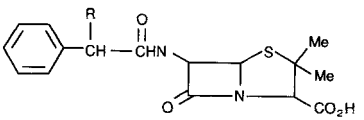
(187) $R^1 = R^2 = Cl$

(188) $R^1 = H$ $R^2 = N_3$



(189)R = OMe

(190)R = N_3

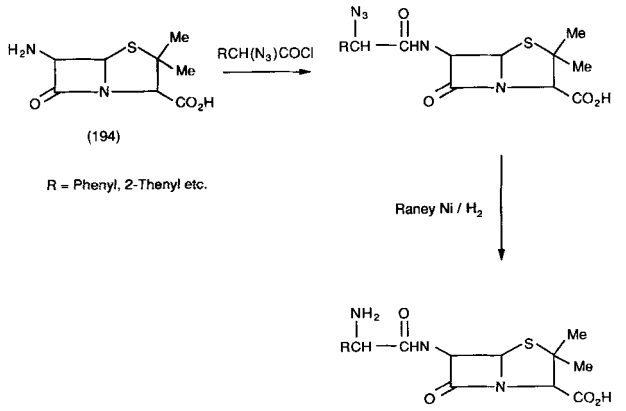


(191) R = H

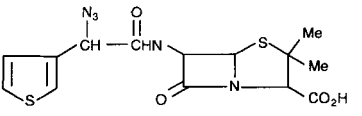
(192) R = NH_2

(193) R = N_3

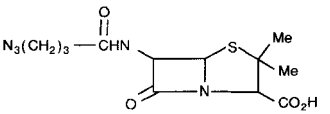
conversion to the azide, with subsequent azido group reduction furnishing the target aminopenicillins in good yield (*Scheme 3.6*). However, in antimicrobial growth-inhibitory studies several of the azido-substituted intermediates including α-azidothenylpenicillin (195), γ-azidopropylpenicillin (196) and, most notably, the α-azidobenzyl derivative (193) were found to exhibit *in vitro* activity comparable with benzylpenicillin (penicillin G) (191) against *Staphylococcus aureus* and *Escherichia coli.*



*Scheme 3.6. Application of the azido substituent in the synthesis of β-*lactams.



(195)

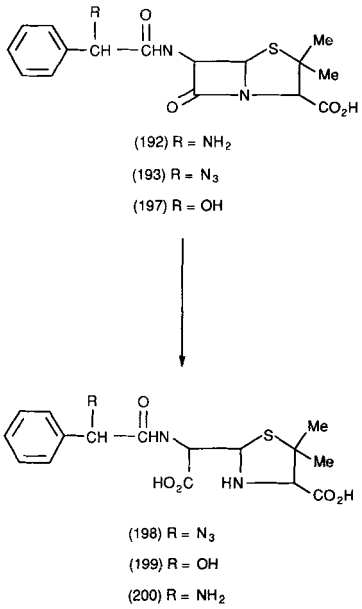


(196)

Subsequent studies confirmed that azidocillin exhibited a spectrum of antibacterial activity similar to the parent benzylpenicillin [253], but like phenoxymethylpenicillin (penicillin V), was acid-stable and readily absorbed following oral administration to patients [254]. Moreover, the drug was more active *in vitro* than phenoxymethylpenicillin against *Haemophilus influenzae*, *Bordetella pertussis* and certain streptococcal strains, and compared favourably with ampicillin against these bacteria [255, 256]. Clinically, azidocillin has been used successfully in the treatment of whooping cough in children, and is reportedly effective in chronic bronchitis [255, 257]. Favourable therapeutic results have also been reported for several other diseases including tonsilitis, otitis and scarlet fever [248, 258], and the incidence of side-effects recorded in a total of 1072 patients receiving the drug, was diarrhoea (3.1%), nausea (0.5%) and skin rashes (1.1%) [258]. The azide has also been used as a feed supplement for the prophylaxis of mastitis in cattle [249].

Pharmacokinetic studies conducted with [^{35}S]-azidocillin in rats and dogs [259] indicated that the drug was rapidly absorbed from the gastro-intestinal tract after oral dosing, with plasma concentrations peaking after 30 min and 1 h, respectively, although absorption in the dog appeared to be less efficient than in man. Autoradiographic studies in mice suggested that azidocillin exhibited a distribution pattern reminiscent of benzylpenicillin. The azidopenicillin was extensively metabolized in both rat and dog with at least two biotransformation pathways being identified, and similar patterns of metabolites were detected in the urine and bile of each species (*Scheme 3.7*). In common with other penicillins, hydrolytic cleavage of the β-lactam ring to afford the corresponding penicilloic acid congener (198) appeared to represent the major pathway of metabolism. Significant quantities of the azide bioreduction products α-aminobenzylpenicillin (ampicillin) (192) and its penicilloic acid (200), were also detected by t.l.c, together with minor metabolites thought to be α-hydroxybenzylpenicillin (197) and the appropriate ring-opened metabolite (199). A similar metabolite pattern was also observed after incubation of ^{35}S-radiolabelled azidocillin with rat liver slices, although t.l.c was again the only characterization method employed.

Comparative pharmacokinetic studies in healthy human volunteers given azidocillin orally (tablet or suspension) or by injection (i.v. or i.m.), demonstrated that while plasma concentrations were higher following parenteral administration, the drug exhibited good bioavailability by both routes of administration, regardless of the formulation [258]. The serum half-life of the drug, 0.6–0.7 h after i.v. administration and 0.6–1.1 h following oral dosing, was of the same order as that for ampicillin, and serum concentrations of azidocillin were higher than those achieved with



Scheme 3.7. Metabolism of azidocillin (193).

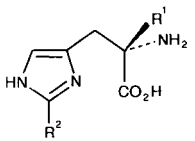
comparable doses of phenoxymethylpenicillin or ampicillin. Urinary recovery of the azidopenicillin was also markedly higher than that of several other penicillins including phenoxymethylpenicillin and ampicillin, suggesting that the high concentrations of drug in the urine may be beneficial in the treatment of certain urinary tract infections. Michel *et al.* compared the serum concentrations of azidocillin (500 mg) and ampicillin (250 mg) given orally to children, and detected similar serum-antibiotic concentrations of the two drugs at these doses. However, the half-life of azidocillin (0.54 h) was considerably shorter than that of ampicillin (1.39 h) in this study [260].

Miscellaneous anti-infective azide derivatives

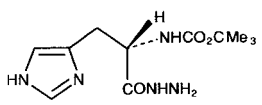
A number of histidine analogues, including 2-azido-L-histidine (201), have been evaluated by the NIH as part of a programme directed towards the development of new antimalarials for the prophylaxis and treatment of drug-resistant *Plasmodium falciparum* malaria [261]. Asexual *P. falciparum* parasites have a much higher histidine content than mammalian cells, and several histidine-rich proteins are thought to be functionally important in

the erythrocyte-stage of the parasite life-cycle. Thus, histidine analogues were investigated as possible amino acid antimetabolites of parasite growth, in the expectation that quantitative differences in cellular histidine content between host and parasite might lead to selective toxicity. Antimalarial activity was determined *in vitro* using infected blood and the effect of each histidine analogue (0.125 and 1.0 mM) was quantified, following incubation for 22 h, by Giemsa staining and microscopic analysis of the morphological development of immature ring-forms of the parasite. Inhibitor potency was correlated with the appearance of condensed or pycnotic parasites rather than mature trophocytes. In addition, the effects of histidine analogues on the incorporation of [^{3}H]-isoleucine into erythrocyte hot-acid-insoluble material, a measure of malarial protein synthesis, was determined.

Of the twenty-five compounds screened, the 2-substituted histidine analogues (201)–(203), together with α-methyl-L-histidine (206) and the hydrazide derivative (207) were found to inhibit *P. falciparum* growth and protein synthesis at 1.0 mM, while the 2-fluoro (202) and 2-iodo (203) analogues were also inhibitory at 0.125 mM, compound (202) proving the most potent of the compounds evaluated. Unfortunately, 2-bromo-L-histidine and 2-chloro-L-histidine (204) and (205) were not available for comparison with the 2-azido amino-acid (201). The exact mechanism of



(201) $R^1 = H$ $R^2 = N_3$
(202) $R^1 = H$ $R^2 = F$
(203) $R^1 = H$ $R^2 = I$
(204) $R^1 = H$ $R^2 = Br$
(205) $R^1 = H$ $R^2 = Cl$
(206) $R^1 = Me$ $R^2 = H$

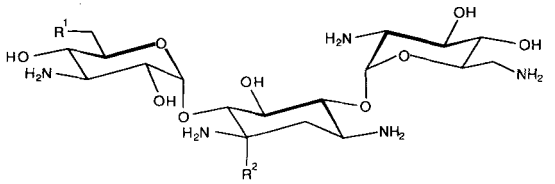


(207)

action of these antimetabolites was not elucidated but a process involving the deleterious misincorporation of the amino-acid analogues into proteins was suggested. Additionally, in a separate experiment 2-fluoro-L-histidine (202) and 2-azido-L-histidine (201) at respective concentrations of 0.125 and 1.0 mM, selectively inhibited the uptake of [^{3}H]-histidine into a histidine-rich protein, although whether this reduction reflected decreased protein synthesis, antimetabolite incorporation or both processes was unclear. Previous information regarding the *in vivo* toxicity of the fluoro derivative

(202) in mice indicated an LD_{50} of 250 mg/kg after 5 days, with acute toxic effects being observed at lower doses [262].

In order to investigate structure-toxicity relationships in the aminoglycoside antibiotics, a group in Belgium has synthesized a series of derivatives of kanamycin B (208), modified by replacement of the 6″-hydroxyl group with a variety of substituents [263]. Of the thirty-three compounds prepared, only those with relatively small 6″-substituents, including the azido, bromo and chloro derivatives (209)–(211) exhibited a spectrum of antibacterial activity virtually identical to the parent aminoglycoside (208), although none



(208) R^1 = H R^2 = H
(209) R^1 = N_3 R^2 = H
(210) R^1 = Br R^2 = H
(211) R^1 = Cl R^2 = H
(212) R^1 = H R^2 = CH_2OH
(213) R^1 = N_3 R^2 = CH_2OH
(214) R^1 = Cl R^2 = CH_2OH
(215) R^1 = NHAc R^2 = CH_2OH

showed activity against kanamycin B resistant strains (*Table 3.19*). Of these, only 6″-chlorokanamycin B (211) was perhaps slightly less potent than kanamycin B as an inhibitor of lysosomal phospholipase A_1 (PLA_1), a measure of the undesirable oto- and nephro-toxic potential of aminoglycosides, whereas the azide (209) was approximately equipotent with the parent compound against this enzyme [264].

For gentamycin derivatives, the introduction of an axial hydroxymethyl substituent at C-1 in the 2-deoxystreptamine moiety reportedly ameliorates nephrotoxicity and confers protection against inactivation by bacterial enzymes. The Belgium team investigated a similar modification in the kanamycin B series [265]. As expected, 1-*C*-(hydroxymethyl)kanamycin B (212) proved equipotent with the parent aminoglycoside (208) against kanamycin B-sensitive bacteria and the introduction of a 6″-azido, 6″-chloro, or 6″-acetamido group (213)–(215), did not reduce antibacterial activity (*Table 3.19*). However, the introduction of a 1-*C*-(hydroxymethyl)

Table 3.19. ANTIBACTERIAL ACTIVITY OF KANAMYCIN B DERIVATIVES AGAINST REPRESENTATIVE KANAMYCIN B SENSITIVE ORGANISMS [263–265]

Compound	*Minimum inhibitory concentration (mg/L) Organism and strain no.*[a]		*Inhibition of PLA_1 activity IC_{50} (μM)*
	Staph. aureus (ATCC 25923)	*E. coli (ATCC 25922)*	
208	0.5	1.0	110 ± 11[b]
209	0.25	1.0	123 ± 11
210	0.25	1.0	129 ± 16
211	0.25	0.5	89 ± 12
212	0.25	0.5	79 ± 12
213	0.5	1.0	95 ± 11
214	0.5	1.0	105 ± 8
215	1.0	4.0	119 ± 20

[a]Full details of activity against other organisms have been published [263, 265];
[b]Standard deviation.

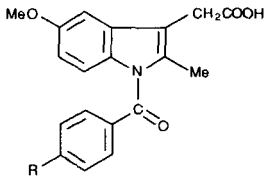
substituent was unsuccessful in protecting the kanamycin B derivatives from destruction by bacterial enzymes, with none of these exhibiting activity against kanamycin B-resistant organisms. Surprisingly, 1-*C*-(hydroxymethyl)kanamycin B (212) was more potent than kanamycin B as an inhibitor of lysosomal PLA_1, suggesting that the former should have an even greater potential to elicit ototoxicity, and although PLA_1-inhibitory activity was reduced with the 6″-substituted analogues (213)–(215), this was not to the extent of offering any advantages over kanamycin B.

ANTI-INFLAMMATORY AZIDES

Zidometacin

In a systematic search for novel analogues of the anti-inflammatory indole-3-acetic acid derivative indomethacin (216), scientists at the Pierrel Laboratories in Milan reasoned that substitution of an azido group for the 4-chloro substituent on the *N*-benzoyl ring might represent an effective bioisosteric replacement, and thus confer interesting biological properties to the molecule [266]. 1-(4-Azidobenzoyl)-5-methoxy-2-methyl-1*H*-indole-3-acetic acid (zidometacin) (217) was synthesized and evaluated for its

pharmacological and toxicological properties in comparison with the parent compound (216), and the established anti-inflammatory drugs phenylbutazone (PBZ) and aspirin. Preliminary studies with oral zidometacin indicated that the drug was approximately equipotent with indomethacin as an



(216) R = Cl

(217) R = N_3

anti-inflammatory agent in the carrageenan-induced rat paw oedema model, and significantly more potent than phenylbutazone or aspirin (*Table 3.20*). In common with indomethacin, the azidoindole was also found to exhibit analgesic and antipyretic activity, but more importantly the acute oral toxicity in mice was approximately 20-fold lower than that of the parent drug, with no evidence of autonomic or CNS involvement. Crucially, zidometacin proved some 5-fold less ulcerogenic than indomethacin in a study with fasted rats sacrificed 16 h after oral administration of either drug at five dose levels, and assigned ulcerogenic doses of 50% (UD_{50}) by subjective scoring of gastric lesions.

A subsequent in-depth investigation of the pharmacological properties of zidometacin, in both acute and sub-acute models of inflammation, confirmed an activity profile comparable with indomethacin, and a potency ratio of 1 to 1/3 depending on the particular test, but that the drug was indeed significantly less toxic and ulcerogenic than indomethacin [267]. The same group subsequently demonstrated that doses of zidometacin found to completely inhibit the production of PGE_2-like compounds in rat inflammatory exudates, only partially reduced the levels of these compounds in gastric mucosa [268]. However, indomethacin showed no such tissue selectivity, implying that the contrasting ulcerogenic potential of the two drugs may reflect differences in their respective activities as inhibitors of gastric cyclo-oxygenases. Pharmacokinetic and bioavailability parameters were determined for zidometacin following the administration of a single oral dose, either as a solution of the sodium salt (100 mg) or as capsules (100 or 200 mg), to nine healthy volunteers [269]. The drug was well absorbed orally, giving identical plasma and urine levels regardless of the formula-

Table 3.20. PHARMACOLOGICAL ACTIVITY AND TOXICITY OF ZIDOMETACIN (217) AND OTHER ANTI-INFLAMMATORY DRUGS [266, 267]

Compd.	*Pharmacological assay (ED_{50} mg/kg p.o. unless indicated otherwise)*				
	Carrageenan Oedema	*PBQ[a]-induced algesia*	*Yeast-induced pyrexia*	*Acute toxicity in mice (LD_{50})*	*Ulcerogenic activity in rats (UD_{50})[b]*
(216)	3.8 (1.5–9.6)[c]	4.1 (2.5–6.6)	7.9 (3.0–20.1)	13.5 (7.7–23.6)	13 (10.4–16.3)
(217)	4.7 (2.2–9.7)	11.4 (7.5–17.3)	7.0 (2.0–27.0)	250 (171.2–363.9)	70 (54.7–89.6)
PBZ[d]	50.5 (22.5–113.0)	220 (146–330)	–	700 (526–931)	–
Aspirin	94.3 (46.4–191.6)	130 (87–192)	–	1000 (625–1600)	–

[a]Phenylbenzoquinone;
[b]Dose inducing an ulcer score of 50%;
[c]95% confidence limits;
[d]Phenylbutazone.

tion, and proved very similar to indomethacin in undergoing elimination with a half-life of approximately 3 h, extensive glucuronidation being observed. Clinical studies to date have been encouraging with zidometacin reportedly proving less irritating to the gastrointestinal tract and as effective as indomethacin in the management of osteoarthritis of the hip [270]. Side-effects following oral administration of the drug included dizziness and nausea, neither of which necessitated cessation of treatment [271].

AZIDES AFFECTING CNS FUNCTION

Azidomorphine and derivatives

An early example of the application of the azido group in drug design is azidomorphine (6-deoxy-6-azidodihydroisomorphin) (72), one of a series of potent azido-substituted opiate derivatives synthesized in Budapest [272, 273]. Preliminary pharmacological studies with azidomorphine and related semisynthetic morphine alkaloids including azidocodeine (218) [274–276], demonstrated that replacement of the 6-hydroxy group of dihydroisomorphine by an azido substituent profoundly modified biological activity compared with the parent opiate, and in particular appeared to enhance

analgesic potency without concomitantly increasing the tolerance and dependence capacity of the drug [277, 278]. Thus, azidomorphine and the closely related congener 14-hydroxyazidomorphine (6-deoxy-6-azidodihydro-14-hydroxyisomorphine) (219) were between 14 and 300 times more potent than morphine in animal models, and as such are among the most effective semi-synthetic morphine alkaloids to date. The bioisosteric

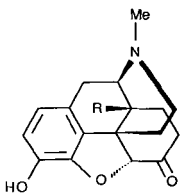
(72) R^1 = Me R^2 = H R^3 = H

(218) R^1 = Me R^2 = H R^3 = Me

(219) R^1 = Me R^2 = OH R^3 = H

(220) R^1 = $CH_2(C_3H_5)$ R^2 = H R^3 = H

relationship between azido and chloro functionalities does not appear to apply in this series, with 6-chloro substituted morphine derivatives exhibiting greater CNS-depressant activity and toxicity than the parent compounds. Knoll and coworkers investigated the pharmacological activity of 14-hydroxyazidomorphine in comparison with morphine, azidomorphine and two structurally related derivatives, hydromorphone (221) and oxymorphone (222), in mice, rats and monkeys [279]. The two azidomorphine derivatives (72) and (219) were the most active of the series, proving equipotent as analgesics and exhibiting the same low tolerance and dependence potential. However, 14-hydroxyazidomorphine was a more



(221) R = H

(222) R = OH

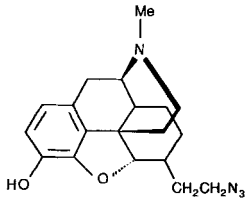
potent antitussive than azidomorphine and appeared to be significantly less toxic in rats and mice.

Knoll and Zsilla proposed that the azido substituent of azidomorphine may be responsible for the low tolerance capacity exhibited by the drug [280]. Daily administration of escalating doses of morphine to rats for four weeks led to a profound reduction of hepatic *N*-demethylase activity in liver homogenate preparations from treated animals, and this effect was completely reversed two weeks after withdrawal of drug treatment. In contrast, animals treated identically with azidomorphine or azidocodeine experienced only a transient decline in *N*-demethylase activity, with metabolic activity being restored upon continued administration of the drugs. Based on an earlier hypothesis that opiates interact with the N-demethylase enzyme and central morphine receptors similarly [281], the relative enzyme-inhibitory activities of morphine and the azidomorphine derivatives were thought to parallel their potency as inactivators of the morphine receptors, and hence their potential to induce tolerance. The authors contend that the C-6 hydroxyl group of morphine plays an important role in receptor (and enzyme) inactivation, and that the introduction of an azido substituent abolishes this effect whilst maintaining the intrinsic activity essential for analgesic activity.

In certain animal models, the evidence for a dissociation of analgetic and dependence-producing properties is equivocal. Hill *et al.* [282] compared the effects of morphine and azidomorphine in a panel of animal screens and concurred that in terms of analgesic activity, azidomorphine was more potent than the parent opiate in all tests conducted, although differences in activity between the two drugs were much less pronounced by the oral route than following parenteral administration. However, the programmed administration of azidomorphine to rhesus monkeys over a period of nine weeks invoked physical dependence, and the animals developed a profound opiate-like dependence to the drug after three weeks of self-administration, leading the authors to conclude that, at least in these animals, equianalgesic doses of azidomorphine are no less likely to induce dependence than morphine.

The pharmacokinetic characteristics of a number of radiolabelled (^{14}C and ^{3}H) azidomorphines have been determined in rodents [283]. The rate of gastrointestinal absorption of azidomorphine was approximately equivalent to that of morphine, whereas azidocodeine (218), 14-hydroxyazidomorphine (219) and azidoethylmorphine (223) were all found to undergo more rapid absorption. Peak CNS concentrations of all of the azidomorphine derivatives were uniformly higher than that achieved with morphine in these studies, and, in rats, the drugs were eliminated principally by the urinary

route with an elimination half-life of approximately 4 h, some 90% of the radioactivity being cleared within 48 h. The metabolic fate of azidomorphine in the rat [87] and in man [88] has been discussed previously.



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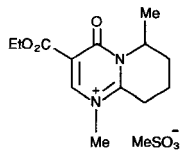
Studies in human subjects have shown that azidomorphine is between 10-and 50-fold more potent than morphine, and that the drug elicits the pharmacological effects characteristic of a typical morphine-like drug, including miosis, euphoria, morphine-type subjective effects and suppression of the morphine abstinence syndrome [284]. Clinical trials conducted with healthy male volunteers, who were given 4 μg/kg of parenteral azidomorphine (i.v. or i.m.), indicated that the drug caused less circulatory and respiratory depression than equianalgesic doses of morphine, and that the incidence of side-effects invariably associated with morphine, for example, nausea and vomiting, was markedly less pronounced with azidomorphine [285]. Intramuscular azidomorphine (4 μg/kg) was more effective in severe than mild or moderate models of experimental pain, and analgesia was most pronounced with ischaemic pain rather than that elicited by electrical stimulation of the earlobe or tooth pulp. In patients suffering chronic intractable pain, a combination of 0.5 mg azidomorphine and 15 mg of the homopyrimidazole non-narcotic analgesic rymazolium mesylate (probon) (224) [286] reportedly achieved total pain relief without euphoria or significant respiratory depression. None of the patients exhibited acute abstinence syndrome after administration of the opiate antagonist nalor-



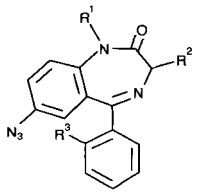
(224)

phine, and the author concludes that azidomorphine is indeed less likely to cause tolerance and physical dependence in man [287, 288].

One other azidomorphine derivative of note is *N*-cyclopropylmethylnorazidomorphine (220) which has proven a useful pharmacological research tool for discriminating between opiate receptor sub-types [289]. Unlike morphine and azidomorphine which are opiate B receptor agonists, the cyclopropylmethyl congener acts as an antagonist at these receptors but stimulates opiate A receptors, and the drug was employed in experiments to differentiate between opiate A and opiate B receptor types based on this differential activity [290].

Azidobenzodiazepines

As part of a systematic search for benzodiazepines exhibiting unusual pharmacological properties, scientists at Hoffman-La Roche synthesized a number of novel azido-substituted benzodiazepine derivatives [291]. The introduction of an azido group in the 7-position of 5-phenyl-1,4-benzodiazepines afforded a series of compounds (225)–(231) which included the 7-azido analogues of diazepam and oxazepam (228) and (229). The azido pharmacophore endowed potency and a spectrum of biological activity comparable with the corresponding 7-halo and 7-nitro analogues, and far superior to the 7-amino derivatives. All of the azides prepared were

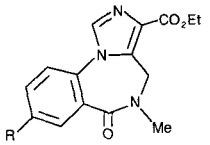


(225) R^1 = H R^2 = H R^3 = H
(226) R^1 = H R^2 = H R^3 = CF_3
(227) R^1 = H R^2 = H R^3 = Cl
(228) R^1 = Me R^2 = H R^3 = H
(229) R^1 = H R^2 = OH R^3 = H
(230) R^1 = Me R^2 = H R^3 = F
(231) R^1 = $(CH_2)_2OH$ R^2 = H R^3 = H

active *in vivo*, following oral administration, in murine and feline assays for sedative, muscle relaxant, anxiolytic and anticonvulsant efficacy, with

compounds (228)–(231) exhibiting activity comparable to diazepam and chlordiazepoxide.

Probably the most intriguing and controversial CNS-active azide derivative to be investigated is another Hoffman-La Roche compound Ro15-4513 (233), an analogue of the benzodiazepine receptor antagonist flumazenil (Ro15-1788) (232). Not surprisingly, this compound was initially



(232) R = F

(233) R = N_3

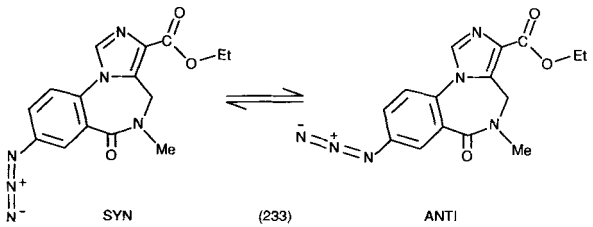
synthesized as a photoaffinity labelling reagent, proving useful for mapping brain γ-aminobutyric acid (GABA)-benzodiazepine receptor sites [292, 293]. However, Skolnick, Paul and coworkers [294] made the important observation that Ro15-4513 was also a potent and selective antagonist of the ethanol-induced (GABA) receptor-mediated uptake of [^{36}Cl]-chloride into isolated rat brain vesicles, implying that ethanol may impart many of its neuropharmacological effects *via* central GABA receptors. Furthermore, administration of Ro15-4513 to ethanol-intoxicated rats rapidly reversed intoxication, while pretreatment with the azide prior to ethanol administration prevented the development of intoxication and other related behavioural changes in the animals with no apparent ill-effects. These astonishing results led to much speculation concerning the obvious ethical and legal implications of developing the benzodiazepine for clinical use as an ethanol antagonist [295, 296]. Interestingly, the antagonistic effects of Ro15-4513 on ethanol were completely abolished by benzodiazepine receptor antagonists, including flumazenil, but not by other benzodiazepine receptor reverse agonists, suggestive of a novel interaction of Ro15-4513 with the GABA receptor-coupled chloride-ion channel complex. That substituting the 8-fluoro substituent of flumazenil by an azido group brings about such a profound change in the biological activity of the imidazobenzodiazepine, suggests that the azido pharmacophore makes important interactions with the benzodiazepine receptor.

The demonstrated activity of Ro15-4513 as an ethanol antagonist has prompted numerous behavioural and biochemical investigations designed

to elucidate the psychopharmacology of ethanol with the importance of GABA-receptor involvement proving particularly contentious, and the results of these studies have been reviewed [297–300]. More recent animal studies suggest that the compound only partially blocks the effects of alcohol, reverses the behavioural effects of barbiturates and diazepam, and additionally exhibits anxiogenic and proconvulsive activities reversible by flumazenil, consistent with activity as a partial reverse agonist (negative allosteric modulator) of the benzodiazepine receptor [301–303]. It is proposed that this intrinsic activity probably militates against the potential therapeutic utility of Ro15-4513 in man.

Recent studies have demonstrated that Ro15-4513 and related imidazodiazepines exhibit a high affinity for the 'diazepam-insensitive' (DI) isoform of benzodiazepine receptors, a distinct receptor subtype with constrained ligand binding requirements compared with other CNS benzodiazepine receptors [304]. This observation has led to the proposal that the ability of these compounds to reverse some of the effects of ethanol may involve DI benzodiazepine receptors [305], and has prompted further investigations regarding ligand requirements for selective, high affinity binding to these receptors. Very recently, QSARs have been determined for affinity and selectivity of binding of 47 benzodiazepines to both DI and diazepam-sensitive (DS) receptor subtypes [306]. Substitution at the 8-position of the imidazodiazepine appears to be a requirement for high affinity binding to DI receptors and also enhances selectivity for the DI site, while ligand affinity was found to increase with the size of the 3-ester function with maximal activity being observed with a t-butyl ester. Receptor volume mapping about the structure of Ro15-4513 showed favourable binding regions corresponding to the 3-ester position and also displayed a receptor pocket occupied efficiently by the 8-azido group consistent with the high affinity binding to DI receptors conferred by this substituent. Of particular interest were the results of studies to determine the active conformation of Ro15-4513, where both the 3-ester and 8-azido functions may adopt low energy *syn* or *anti* conformations (*Scheme 3.8*). Whereas the active conformation of the 3-ester group appears to be *anti* for both the DI and DS receptors, the DI active conformation of the 8-azido substituent was found to be *anti*, while binding to the DS receptors would almost certainly require that the azido group adopt the presumably less favourable *syn* conformation. Thus, the azido substituent appears to play an important role in the selective binding of Ro15-4513 to DI benzodiazepine receptors.

The brain distribution and binding of Ro15-4513 has been evaluated recently by autoradiography and positron emission tomography (PET) techniques using [^{11}C]-labelled material. Autoradiographic analysis of



Scheme 3.8.

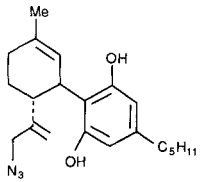
human post-mortem brain sections *in vitro* demonstrated that the azidobenzodiazepine accumulated predominantly in the neocortex region, with high levels being detected in the basal ganglia and cerebella cortex, and while flumazenil or clonazepam inhibited binding in cerebral regions, significant levels of radioactivity remained in the cerebellum [307]. Similar results were also obtained with PET scans conducted *in vivo* with monkeys in this study. Japanese researchers compared the kinetics of distribution of [^{11}C]-Ro15-4513 and [^{11}C]-flumazenil in human volunteers by PET scanning and observed that whereas the labelled flumazenil was distributed almost homogenously throughout the cortex region, the highest concentrations of [^{11}C]-Ro15-4513 were detected in the frontal and temporal cortex and the hippocampus regions of the brain [308]. Differences in the localization of the labelled ligand were thought to largely reflect the relative affinities of each of the benzodiazepine derivatives for different regions of the brain.

Miscellaneous CNS-active azides

A number of nitrogen-containing derivatives of Δ^8-tetrahydrocannabinol (Δ^8-THC), synthesized as possible cannabinoid antagonists and to further elucidate SARs for this class of compound, included the 10-azidocannabidiol (234). Pharmacological studies with mice, utilizing a panel of behavioural models including locomotor activity, antinociception, and induction of catalepsy or hypothermia, demonstrated that the introduction of an azido substituent at C-10 was dystherapeutic, and (234) was essentially inactive except for the production of respiratory depression concomitant with toxicity [309].

Tunnicliff and Ngo have reported that the aryl azide 4-(4′-azidobenzoimidylamino)butanoic acid (ABBA) (235) not only inhibits the binding of the brain neurotransmitter GABA to its transporter [310], but is also a potent competitive inhibitor of the uptake of GABA by brain synaptosomes [311].

GABA-neurone disfunction in the brain is implicated in a number of disorders, including epilepsy and Huntington's disease, and increasing the effective concentration of the neurotransmitter by employing agents which, by preventing GABA uptake from the synaptic gap indirectly function as GABA-mimetics, represents a therapeutic alternative to the use of GABA agonists. The uptake of [^{3}H]-GABA was determined in a synaptosomal preparation isolated from rat brain homogenate by sucrose-density centrifugation, and radioactivity was measured in the synaptosomal pellet following incubation for 3 min at 37°C with the appropriate concentration of radiolabelled GABA, when the reaction was terminated by centrifugation. Both a high affinity (K_m = 3 μM) and a low affinity (K_m= 75 μM) uptake system was observed in these studies, each being competitively inhibited by ABBA with K_i values of 8 and 16 μM, respectively. The azide derivative was also found to interfere equally (K_i = 9 and 22 μM) with the



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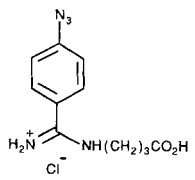
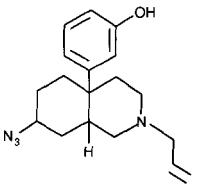


(235)

respective high and low affinity components (K_D = 1.9 and 66 μM) of the sodium-dependent binding of GABA to synaptic membranes, implying that association with the membrane precedes the uptake process. It is suggested that in addition to representing a potentially useful photoaffinity reagent, ABBA might exhibit direct therapeutic activity as a GABA-mimetic agent although this does not appear to have been established to date.

Several other azido-substituted compounds have been reported to exhibit CNS-activity [312]. Sandoz have patented the decahydroisoquinoline derivative (236) as a sedative [313], while hypnotic activity has been claimed for all of the isomers of 1,4:3,6-dianhydro-2,5-diazido-2,5-dideoxyhexitol (237) [314]. The hypnotic and spasmolytic activity of the 5-azidobarbituric acid (238) has also been reported [315].



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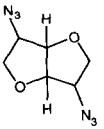

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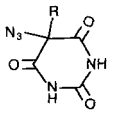


(238) R = Alkyl or Phenyl

AZIDES INFLUENCING CARDIOVASCULAR ACTIVITY

Diuretic azidopyrimidines

A series of novel substituted 4-azidopyrimidines exhibiting potent diuretic activity has been reported by G.D. Searle. Some thirty-four compounds were evaluated in rats, in comparison with hydrochlorothiazide and spironolactone, for general diuretic and anti-aldosterone activity, respectively, and biological activity varied considerably within the series (*Table 3.21*) [316]. Optimal diuretic activity was observed in 2-aminopyrimidines bearing a 4-azido substituent and a phenyl group at the 6-position, and the requirement for a primary amine at the 2-position was confirmed by the overall reduction in potency observed on introducing a secondary amino moiety (2-NHMe). No mention is made of the fact that this series of 4-azidopyrimidines presumably exists in tautomeric equilibrium with the corresponding tetrazoles as discussed previously, with the tetrazole tautomers predominating. A pronounced dystherapeutic effect was also seen on substitution of the 4-azido group with amino or hydroxyl, whereas replacing the phenyl ring by a 2-thiophenyl isostere or introducing substituents in the 6-aryl position generally dramatically lowered diuretic activity.

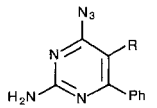
Structure–activity relationships at the 5-position were more interesting and enabled some differentiation between general diuresis and aldosterone antagonist activity. Thus, while maximal general diuretic activity in the series was obtained with the 5-ethoxyethyl derivative (239), this azidopyrimidine proving more potent than hydrochlorothiazide and also spironolac-

Table 3.21. GENERAL DIURESIS AND MINERALOCORTICOID ANTAGONIST ACTIVITY OF 2-AMINO-4-AZIDO-5-SUBSTITUTED-6-PHENYLPYRIMIDINES [316]

Compound	*Diuretic activity*		
	General (%)[a]		*Anti-DCA*[b] *MED*[c] *(mg per animal)*
	Volume	*Na*	
(239)	125	133	0.64
(240)	32	27	0.25
(241)	28	31	0.35
(242)	<0.42	0.57	0.35
Spironolactone	<0.83[d]	<0.83[d]	0.33

[a]Potency relative to hydrochlorothiazide;
[b]Deoxycortisone acetate;
[c]Median effective oral dose for 50% reversal of the urinary log Na/K response to DCA;
[d]Inactive.

tone, the 5-acetonyl derivative (240) was much less active as a mineralocorticoid antagonist but maintained some level of general diuretic activity. A 5-methyl group (241) restored antialdosterone activity to that observed with spironolactone, and similar potency was seen also with a 2-propynyl substituent (242) albeit with a loss of significant general diuretic activity. On



(239) R = $(CH_2)_2OEt$

(240) R = CH_2COMe

(241) R = Me

(242) R = $CH_2C{\equiv}CH$

the basis of these results 2-amino-4-azido-5-(2-ethoxyethyl)-6-phenylpyrimidine (SC-16102) (239) was selected as a clinical candidate and further pharmacological studies confirmed potent oral diuretic activity in the intact and bilaterally adrenalectomized rat, with the drug exhibiting a potency approximately 7-fold that of spironolactone and 2.5-fold that of hydrochlo-

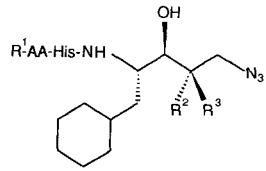
rothiazide [317]. Some evidence in support of ADH antagonist activity in the rat was also adduced for SC-16102 [318] although, interestingly, the pyrimidine was devoid of diuretic activity in dogs. In human subjects SC-16102 (100 mg orally) exhibited potent saluretic activity with a pattern of electrolyte excretion similar to that of the benzothiadiazines, activity peaking after 2 h and persisting for approximately 10 h [319]. Activity is thought to reside principally at the proximal tubule and arise through a different mechanism from hydrochlorothiazide, although the drug appears also to exert some potassium-sparing properties [318, 320]. Adverse effects in these studies were limited to a mild symptomless polymorphonuclear leukocytosis [319].

Azidoglycol renin inhibitors

In an accomplished programme of drug design evolving from previously established SARs for renin inhibitors based on the natural substrate angiotensinogen, medicinal chemists at Abbott Laboratories in Illinois have developed an interesting series of antihypertensive azide derivatives [321]. *Erythro*-glycol analogues of the putative tetrahedral transition state for cleavage of the scissile Leu-Val bond of angiotensinogen are often inhibitory [322], and the group discovered that the entire post-scissile peptide fragment of such inhibitors could be replaced by an azidomethyl group without loss of activity [323]. These two features were incorporated in the design of novel low molecular weight (<600) renin inhibitors of which the azido diol (243) proved the most potent, with an IC_{50} value of 0.40 nM against purified human renin (*Table 3.22*).

The importance of a 2(*S*)-hydroxyl substituent for maximal potency was demonstrated by deletion of the group (244) or inversion of the stereochemistry at C-2 (245), either of these modifications reducing inhibitory activity by approximately 50-fold. Hydrogen bonding within the enzyme active site is thought to account for these observations since ketone (246), with an IC_{50} of 5.4 nM, was more potent than the monohydroxy compound (244) but less so than the diol (243), reflecting the reduced hydrogen bonding ability of a carbonyl compared with a hydroxyl substituent or, alternatively, the unfavourable geometry of an sp^2 centre at this position in the molecule. Elaboration of the 2-hydroxyl group to hydroxymethyl (247) and (248), or methenyl (249) resulted in a significant reduction in activity. The synthetic route developed for the preparation of the azido 1,2-diol pharmacophore of compound (243) is shown in *Scheme 3.9*. Protection of the hydroxyl group with MEM (R = MeO-$CH_2CH_2OCH_2O$) was found to direct epoxidation of the protected allylic

Table 3.22. INHIBITORY ACTIVITY OF AZIDO GLYCOLS AGAINST HUMAN RENIN [321]

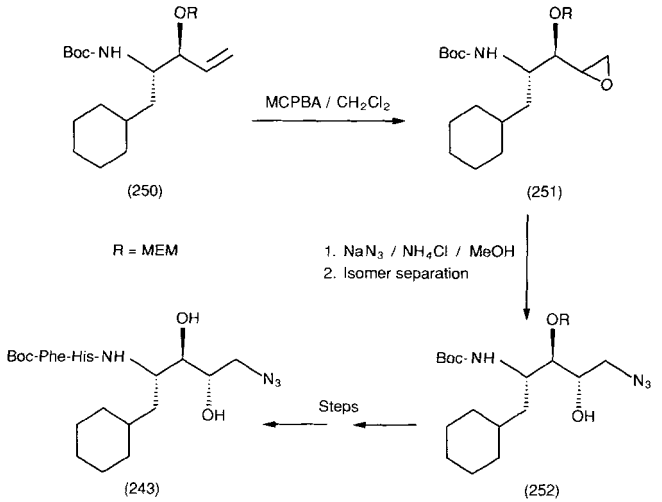


Compd.	R^1	R^2	R^3	AA^a	*Inhibition of human renin IC_{50} (nM)*	
					Purified renal	*Plasma (pH 7.4)*
(243)	$(Me_3)_3CO$	OH	H	Phe	0.40	9.0
(244)	$(Me_3)_3CO$	H	H	Phe	20	–
(245)	$(Me_3)_3CO$	H	OH	Phe	26	–
(246)	$(Me_3)_3CO$	O		Phe	5.4	–
(247)	$(Me_3)_3CO$	CH_2OH	H	Phe	10	–
(248)	$(Me_3)_3CO$	H	CH_2OH	Phe	370	–
(249)	$(Me_3)_3CO$	CH_2		Phe	55	–
(253)	$(Me_3)_2CH$	OH	H	Phe	0.55	3.3
(254)	$(Me_3)_2CH$	OH	H	(Me)Tyr	2.0	10
(255)	morpholin-4-yl	OH	H	(Me)Tyr	4.0	20

[a]Amino acid.

ether (250) 2 : 1 in favour of the requisite 2*S* isomer, and azidation of epoxide (251), followed by chromatographic separation of the resulting diastereomers, furnished the 2(*S*)-azidoglycol (252) which was deprotected and coupled with BOC-Phe-His-OH to give the target renin inhibitor (243). The predicted stereochemistry of intermediate (252) was confirmed by X-ray crystallographic analysis.

Selection of the azidomethyl glycol (243) for development as a possible clinical candidate for oral administration necessitated further structural modification of the *N*-terminus due to the acid lability of the BOC group, and the susceptibility of the Phe-His bond towards cleavage by chymotryp-



Scheme 3.9.

sin. The first of these problems was circumvented without loss of enzyme specificity or potency *in vitro* [324] by substituting the BOC functionality by an isobutyryl group. This derivative (A-62198) (253), was subsequently shown to effect a dose-dependent fall in mean-arterial blood pressure in both sodium deplete and normal monkeys, following administration as an i.v. bolus, and the hypotensive action of the drug was attributed, at least in part, to an inhibition of circulatory renin [325]. The pharmacological activity of A-62198 was also primate-selective in this study, with no hypotensive activity being observed in rats.

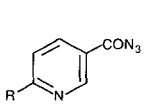
Stability to chymotrypsin was achieved by the previously established strategy of replacing the phenylalanine residue with *O*-methyltyrosine [326], a modification (254) that diminished activity against both purified human renal renin (IC_{50} = 2.0 nM) and the human plasma enzyme (IC_{50} = 10 nm) approximately 4-fold compared with (243). Bioavailability problems associated with (254), and attributed to the extremely low aqueous solubility of the compound, led to further *N*-terminal modification, namely, replacement of the isobutyryl group by morpholin-4-yl (255). The authors had earlier demonstrated that the introduction of this substituent preserves *in vitro* potency [326], and a 100-fold increase in aqueous solubility with (255) was achieved with a concomitant 2-fold loss of renin-inhibitory activity. However, in common with the isobutyryl analogue (254), morpholinourea (255) was devoid of significant *in vivo* antihypertensive

activity following intraduodenal administration (10 mg/kg) to sodium-deplete monkeys. Subsequent bioavailability studies in both an *in vitro* and *in vivo* rat model revealed that while acceptable gastrointestinal transport of (255) occurred *in vivo*, extensive hepatic extraction limited the achievable systemic levels of the drug.

Miscellaneous antihypertensive azides

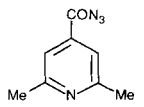
The hypotensive properties of azide ion have been referred to previously [22, 23]. Many simple organic azides, including phenyl azide [327], ethyl azidoformate [312], and alkyl and acyl azides [328] also exhibit antihypertensive activity. Roth and Morphis at Schering investigated the pharmacological properties of an homologous series of linear diazides, $N_3[CH_2]_nN_3$, in a number of pharmacological screens [329]. All of the azides evaluated exhibited antihypertensive activity in hypertensive and normotensive animals after i.v., i.m. or oral administration, with potency declining either side of a maximum observed when n = 7. More detailed biological studies conducted with 1,7-diazidoheptane were not encouraging, a poor correlation being observed between acute toxicity and hypotensive activity in mice. High doses of the diazide administered to unanaesthetized dogs elicited depression, emesis and death, whereas at lower doses a prolonged and significant fall in arterial blood pressure, thought to arise from a peripheral site of action, was observed.

The Schering group also screened twenty-one organic azides for oral hypotensive activity in unanaesthetized rabbits and selected six for further evaluation based on favourable activity compared with sodium azide [330]. All of the organic azides were less toxic than sodium azide, which exhibited toxicity at all hypotensive doses, and the most effective and prolonged reduction in blood pressure was observed with the nicotinoyl and isonicotinoyl azide derivatives (256)–(258). The parent nicotinic and



(256) R = H

(257) R = Me

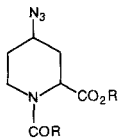


(258)

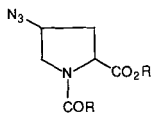
6-methylnicotinic acids were essentially inactive in these studies. A very

similar pattern of hypotensive activity was also observed in anaesthetized dogs and cats, following intraduodenal administration of these azides (1 mg/kg), with a maximum fall in blood pressure (40–60 mm Hg below control) being observed after twenty minutes and continuing for approximately one hour. Werle and Fried [328] proposed that the hypotensive activity of organic azides arises from slow hydrolytic release of azide ion and that, consequently, such compounds should exhibit a similar therapeutic index to sodium azide. However, in this investigation the oral administration (10 mg/kg) of nicotinoyl azides to rabbits produced a reduction in blood pressure comparable with oral sodium azide (3 mg/kg), but without the toxicity observed for the latter compound.

Squibb have patented several azido-substituted pipecolic acid (259) and proline (260) derivatives, thought to act by inhibiting angiotensin-converting enzyme, for the treatment of hypertension [331]. A number of



(259)

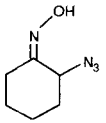


(260)

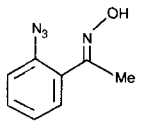
R = Alkyl or Aryl

other azides patented for use in the control of hypertension include 2-azidocyclohexanone oxime (261), 2-azidoacetophenone oxime (262) and β-styrenesulphonyl azide (263), all of which are active orally, and a series of arylsulphonyl azides [332, 333]. The known stability of arylsulphonyl azides towards hydrolysis, together with the high therapeutic indices reported for these compounds indicated that a mechanism of action other than simple hydrolytic release of azide ion was involved, and this was investigated further by Matier *et al.* at Mead Johnson who patented a series of sulphamoyl azides for the control of hypertension [334, 335]. A large number of mono- and di-substituted sulphamyl azides of general structure $R^1R^2NSO_2N_3$ were synthesized, where R^1 and R^2 were H, alkyl, or aryl, or formed part of a piperazine ring [336]. Kinetic studies confirmed the stability of arylsulphonyl azides, including *p*-toluenesulphonyl azide (264), and demonstrated also that disubstituted sulphamyl azide derivatives were extremely resistant to hydrolysis, whereas monosubstituted sulphamyl azides were generally relatively stable in aqueous acid but underwent rapid hydrolysis with release of azide ion at pH 7.0 or above.

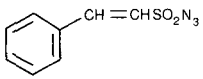
Interestingly, while both classes of compounds provoked a dose-



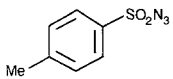
(261)



(262)



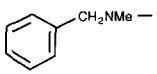
(263)



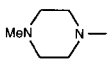
(264)

dependent lowering of systolic and diastolic blood pressure following i.v administration to anaesthetized normotensive dogs, the more labile monosubstituted derivatives were 30–100 times more potent than their disubstituted counterparts, consistent with a mechanism of action entailing release of azide ion. As would be expected, substituting the azido substituent by an aziridinyl, amidino or hydrazino group abolished activity in the series of compounds studied. In order to evaluate the potential oral antihypertensive activity of sulfamoyl azides, selected compounds (265)–(268) were administered intraduodenally using the same animal model. Whereas the disubstituted compounds (265) and (266) were essentially inactive at doses of 1 and 10 mg/kg, administration of 1 mg/kg of the monosubstituted sulphamyl azides (267) and (268) elicited a rapid and pronounced

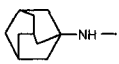
(265) R =



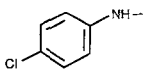
(266) R =



(267) R =



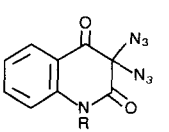
(268) R =



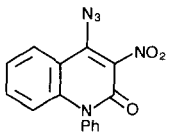
hypotensive effect of approximately 1 h duration, and was attributed to a reduction in both total peripheral resistance and aortic blood flow. The acute oral toxicity of the monosubstituted derivatives in mice also exceeded that of their disubstituted congeners, with respective LD_{50} values of 100–500 and >2000 mg/kg being reported. The authors suggest that the hydrolytically stable disubstituted sulphamyl azides and arylsulphonyl azides suffer enzyme-catalysed hydrolysis *in situ*, or alternatively, are intrinsically hypotensive.

Thrombolytic azidoquinolines

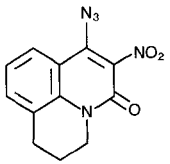
A number of substituted azidoquinoline derivatives, synthesized as potential inhibitors of platelet aggregation, have been reported to exhibit activity *in vitro* [337]. A standard synthetic route involving chlorination of the parent hydroxyquinolines and treatment of the resultant chloroquinoline derivatives with sodium azide in dimethylformamide was employed, as exemplified for compound (271) (*Scheme 3.10*). Compounds were evaluated *in vitro* for their ability to inhibit induction of platelet aggregation in platelet rich plasma by the trigger substances ADP, collagen, platelet-activating factor (PAF) and a prostaglandin H_2 mimetic (U46619). Four of the azidoquinolines (269)–(272) exhibited significant inhibitory activity in these assays with IC_{50} values ranging from approximately 2–7 μM (ADP), 2.5–15.5 μM (PAF), 68–74 μM (collagen) and 48–68 μM (U46619), with all



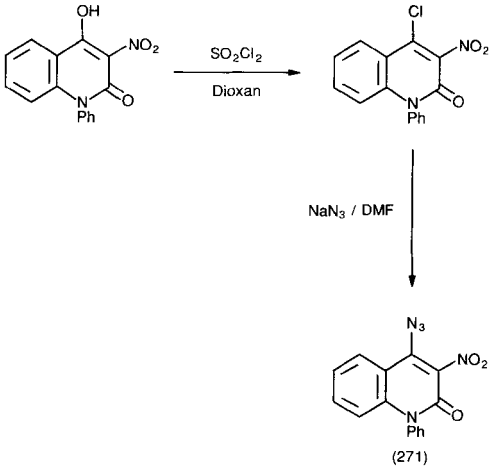
(269) R = H
(270) R = N_3



(271)



(272)

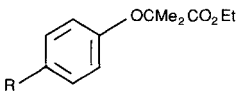


Scheme 3.10.

other compounds giving IC_{50} values in excess of 500 μM in each of the assays. From these results the authors inferred that of the azidoquinolines investigated, maximal inhibitory activity required either a geminal diazido (269) and (270) or a nitro functionality (271) and (272) at the nominal 3-position of the molecule. Although no conclusions were drawn concerning the exact mechanism of action, the compounds were found to inhibit PAF activity in a competitive manner, and the relatively high IC_{50} values observed in the U46619 and collagen-induced aggregation assays indicated that activity through inhibition of the arachidonic acid pathway or binding to a thromboxane membrane receptor was unlikely.

Azidofibrate

Hazards associated with the long-term use of clofibrate (273), an effective lipid-lowering agent, prompted an Italian group to investigate the effect



(273) R = Cl

(274) R = N_3

Table 3.23. INFLUENCE OF AZIDOFIBRATE, CLOFIBRATE AND NICOTINIC ACID UPON ADRENALINE- AND THEOPHYLLINE-INDUCED RAT FAT CELL LIPOLYSIS [338]

Compd. (10^{-4} M)[a]	*Rat fat cell lipolysis (μmol glycerol/10^{-4} cells per 30 min)*	
	Adrenaline (10^{-4} M)	*Theophylline (3×10^{-4} M)*
None (control)	1.108 ± 0.065[b]	0.915 ± 0.013
Clofibrate (273)	0.557 ± 0.039	0.831 ± 0.089
Azidofibrate (274)	0.910 ± 0.041	0.456 ± 0.058
Nicotinic acid	–	0.353 ± 0.077

[a]No effect upon spontaneous glycerol release was observed for any drug at this concentration; [b]Mean ± SEM of data from triplicate incubations in three experiments.

upon toxicity and biological activity, of replacing the 4-chloro group of this drug by an azido substituent [338]. Azidofibrate (274) was evaluated, in comparison with the parent compound, as an inhibitor of adrenaline or theophylline-induced fatty acid lipolysis in fat cells isolated from rat epididymal fat pads. Cells were pre-incubated in the presence or absence of the appropriate drug (15 min, 37°C) prior to initiation of lipolysis by addition of adrenaline or theophylline, and the rates of formation of glycerol after further incubation (30 mins) were quantified and taken as an index of the rate of lipolysis (*Table 3.23*). In the absence of inhibitor, adrenaline induced an 8-fold and theophylline a 7-fold increase in the rate of glycerol release. Clofibrate was found to inhibit adrenaline-induced lipolysis in a dose dependent manner with an IC_{50} of approximately 0.1 mM, whereas azidofibrate was essentially inactive at the same concentration. However, at a maximum concentration of 0.1 mM, clofibrate was without effect on theophylline-induced lipolysis, whereas the same concentration of azidofibrate reduced lipolysis rates by approximately 50%, implying that the antilipolytic activity of the two drugs arises by different mechanisms. Thus, substituting azido for chloro appears to dramatically modify the pharmacological activity of the molecule, and similarities between the azide and nicotinic acid indicated that the two agents might share the same site of action. Regrettably, more comprehensive biological studies do not appear to have been conducted with azidofibrate.

CONCLUDING REMARKS

The emergence of the 3′-azidodideoxynucleosides as effective antiretroviral drugs has, to some degree, alerted the medicinal chemist to the potential utility of the azido substituent as a novel pharmacophore in drug design. Hitherto, awareness of the biological properties of azides was restricted almost entirely to their role as photoaffinity labelling reagents, although the azido group clearly exhibits a number of interesting properties that warrent further investigation. Similarities between azides and the halides, in particular with regard to lipophilicity and electronic character, justifies the consideration of the azido group as an alternative to a chloro or bromo substituent in circumstances where a lipophilic interaction is required, for example on an aromatic ring known to interact with a hydrophobic domain within an enzyme active site. Perhaps of greater interest to the drug designer, however, is the susceptibility of the azido group to undergo metabolic or bioreductive transformation to the corresponding amino group. Azides have the potential to function as lipophilic prodrugs of amines which undergo bioconversion to the parent drug within target tissues. Alternatively, pharmacologically active drugs bearing an azido substituent may undergo inactivation following biotransformation to the more polar amine.

Thus, the incorporation of an azido group into a drug molecule represents a strategy for modulating the biological and pharmacokinetic properties of the drug to therapeutic advantage. Further studies are required to determine structure–metabolism relationships for azido-substituted molecules, to elucidate the enzyme(s) responsible for azide metabolism, and to gain an understanding of the toxicological implications of introducing an azido group into a drug molecule. Finally, the potential role of the azido group as a probe for biological reactions thought to involve the intermediacy of radicals, as demonstrated by the interaction of RNR with N_3UDP (71), merits further investigation. It is hoped that this review may encourage medicinal chemists to regard the azido group as a novel and potentially useful functional group in drug design, and also stimulate further research into the biological properties of azides.

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Progress in Medicinal Chemistry – Vol. 31, edited by G.P. Ellis and D.K. Luscombe

4 Gastric H^+/K^+-ATPase Inhibitors

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INTRODUCTION

MECHANISM OF GASTRIC ACID SECRETION

The oxyntic gland is the secretory unit of the gastric mucosa. The acid-secreting parietal cells are located in the wall of its midsection. In addition to parietal cells, these glands consist of mucous-secreting superficial and neck cells, pepsinogen-secreting chief cells, endocrine, and somatostatin cells [1].

There are three different types of stimulatory receptors on the basolateral membrane of the parietal cells: H_2-receptors, muscarinic receptors and gastrin receptors [1, 2]. Histamine is released by mast cells or mast cell-like cells of the gastric mucosa and reaches the parietal cell in a paracrine manner [3]. The histamine receptor on the parietal cell is of the H_2-receptor subclass. Acetylcholine originates in vagus nerves and cholinergic neurons in the wall of the stomach; its action is neurocrine. Studies with selective muscarinic antagonists in isolated parietal cells indicate that the muscarinic receptor on the parietal cell does not belong to the M_1-receptor subclass, although with selective M_1-receptor antagonists, acid secretion *in vivo* can be inhibited indicating that M_1-receptors are involved in acetylcholine-induced gastric acid secretion possibly within postsynaptic neurons [4]. Gastrin is synthesized and stored in antral G-cells of the stomach and its secretion into the blood is stimulated by amino acids in the gastric juice. Acid at the surface of the antral mucosa inhibits gastrin release and represents the natural break for gastrin release [3]. Neutral pH in the antrum caused by food intake or pharmacologically-induced gastric acid inhibition does not actively stimulate gastrin release from the antral G cells but represents the removal of the natural break of gastrin release. Gastrin mediates its action on the parietal cell in an endocrine manner (*Figure 4.1*). The secretion of gastrin from G cells is associated with suppression of somatostatin release from D cells. Besides gastrin receptors on parietal cells [5], additional locations for gastrin receptors such as histamine-releasing cells mediating acid secretion have been discussed [6]. Regulation of gastric acid secretion is complex and involves central, peripheral and local influences [7]. In addition to the stimulatory receptors, there are also different types of inhibitory receptors on the basolateral membrane of the parietal cell such as receptors for prostaglandin and somatostatin.

The binding of histamine to its H_2-receptor on the parietal cell causes activation of the membrane-bound adenylate cyclase with a corresponding increase in intracellular cyclic AMP levels. The binding of acetylcholine or gastrin to their specific receptors increases intracellular calcium. Increased

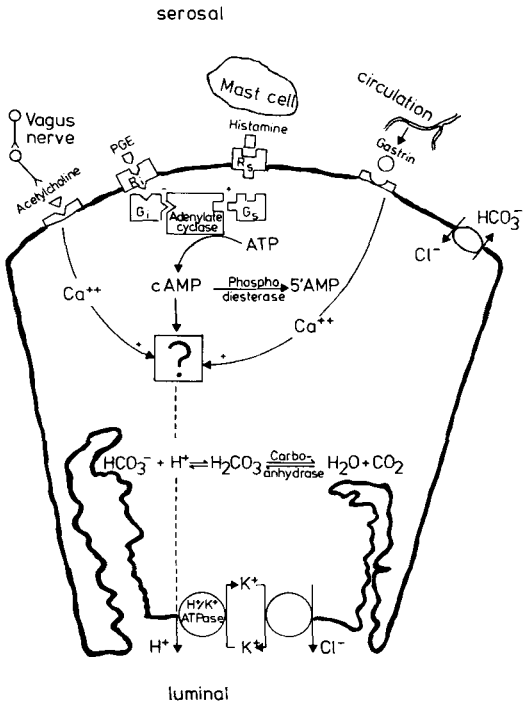

Figure 4.1. Schematic representation of the gastric parietal cell, showing the pathways by which gastric hydrochloric acid is generated and secreted.

levels of both cyclic AMP (histamine) and intracellular calcium (gastrin/acetylcholine) finally cause acid secretion [1] and furthermore there is an interaction between both intracellular pathways [8]. The protons for acid formation are generated intracellularly by the carbonic anhydrase reaction.

The final step of acid secretion is mediated by H^+/K^+-ATPase (E.C. 3.6.1.3.), the so-called gastric proton pump. Shull and Lindgrel reported that this enzyme consists of 1,033 amino acids with a combined molecular weight of 114,012 [9]. It shows about 62% homology of the amino acid sequence of Na^+/K^+-ATPase. Both enzymes are phosphorylated by ATP and potassium binding causes dephosphorylation and a conformational change in the protein. In contrast to Na^+/K^+-ATPase, which is widely distributed in all mammalian cells, H^+/K^+-ATPase is predominantly located at the apical membrane of the parietal cell, although a similar enzyme has

been reported to be located in distal colonic epithelial cells of rabbit [10, 11] and in renal collecting duct segments of rats [12] and rabbits [13].

The apical membrane of the parietal cell forms, under nonsecretory conditions, tubulovesicular structures and, under secretory conditions, secretory cannaliculi. Its morphology depends on the secretory state of the parietal cell [14]. The lumen of the cannaliculi belongs to the extracellular compartment under secretory conditions (*Figure 4.1*) and contains hydrochloric acid with a pH of about 1. H^+/K^+-ATPase exchanges protons for potassium ions across the apical surface. Thereby, the enzyme pumps out the protons against a proton gradient of 1:1,000,000.

A potassium chloride co-transporter must be closely related to the H^+/K^+-ATPase during proton secretion. Potassium and chloride ions move across the apical membrane together with secreted protons (*Figure 4.1*) [15–17]. Potassium is recycled while hydrochloric acid of the gastric juice is formed by chloride ions together with the secreted protons. Stimulation of gastric acid secretion across the apical membrane may predominantly reflect the activation or insertion of an active potassium chloride co-transporter rather than direct activation of H^+/K^+-ATPase [1].

INHIBITION OF GASTRIC ACID SECRETION

Gastric acid secretion can be pharmacologically inhibited by specific antagonists of the stimulatory receptors (histamine-H_2, muscarine-M_1/M_2, gastrin), by agonists to inhibitory receptors (prostaglandin, somatostatin), by carbonic anhydrase inhibitors, and by H^+/K^+-ATPase inhibitors.

In a well-defined pharmacological model, the rat perfused stomach-lumen, acid secretion can be dose-dependently induced by the infusion of histamine, carbachol or gastrin [18]. In this model, cimetidine inhibited histamine- and gastrin-induced gastric acid secretion but had little effect on carbachol stimulation. Atropine caused inhibition only during carbachol-induced gastric acid secretion without causing any inhibitory effects during histamine or gastrin stimulation. Proglumide, a weak but selective antagonist of gastrin and cholecystokinin receptors, only inhibited gastrin stimulation (*Figure 4.2*).

In contrast to the different types of receptor antagonists, omeprazole, the first H^+/K^+-ATPase inhibitor used clinically, causes a comparable inhibition of stimulated gastric acid secretion irrespective of the kind of stimulation (*Figure 4.3*). Acid secretion can be induced in the rat perfused stomach-lumen model by an initial injection of isobutyl-methylxanthine (IBMX, a phosphodiesterase inhibitor) followed by an infusion of forskolin (direct stimulation of adenylate cyclase). This kind of stimulation represents an

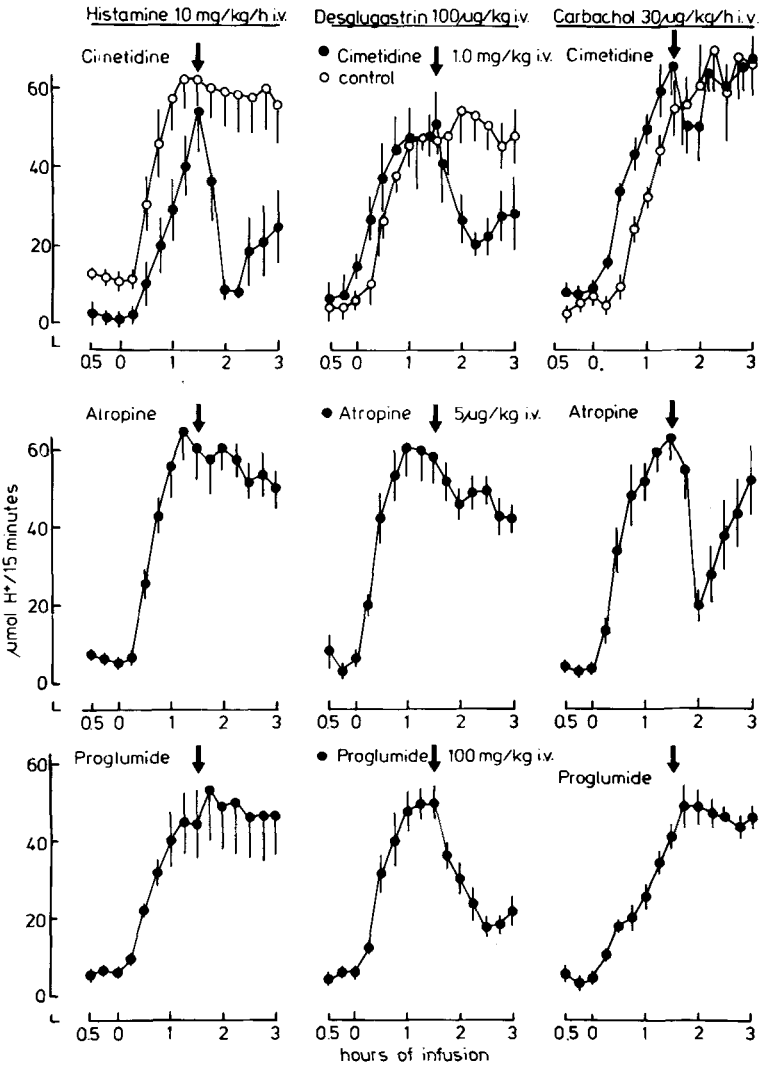

Figure 4.2. Inhibitory profiles of different kind of antisecretory receptor antagonists on stimulated gastric acid secretion in rat perfused stomach-lumen. Desglugastrin represents a gastrin analogue. Values are means +/− S.E.M., n = 4–5 rats.

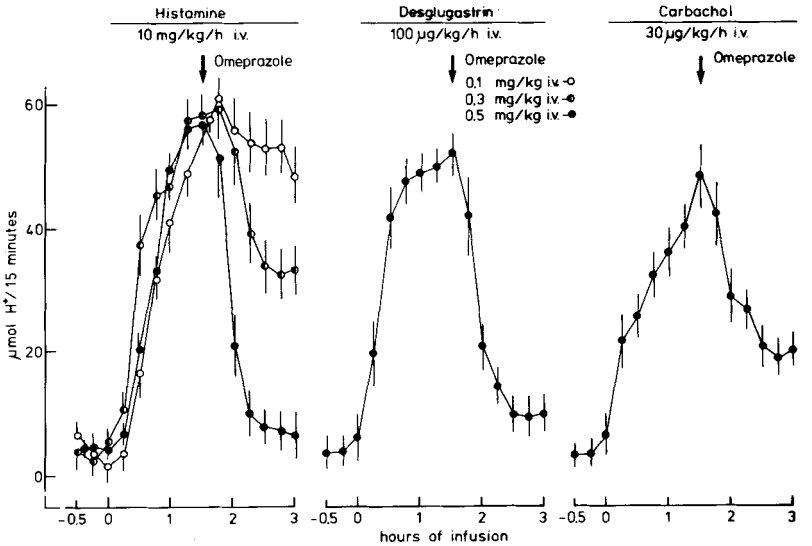

Figure 4.3. Inhibitory profile of omeprazole on stimulated gastric acid secretion in rat perfused stomach-lumen. Values are means +/− S.E.M., n = 4–5 rats.

induction of gastric acid secretion on a sub-receptor level *in vivo* similar to dbcAMP stimulation of acid formation *in vitro* in isolated gastric glands or isolated parietal cells. Omeprazole also inhibits IBMX-forskolin induced gastric acid secretion [18]. The inhibition of gastric H^+/K^+-ATPase, representing the last step of acid formation within the parietal cell, is up to now, the most efficient method of blocking gastric acid secretion. H^+/K^+-ATPase inhibitors can abolish acid secretion stimulated by any secretagogue [19, 20].

THERAPEUTIC VALUE OF GASTRIC ACID INHIBITION

The physiological importance of acid secretion and acid-induced pepsinogen activation is to initiate the digestive process, kill bacteria and other microbes and ensure a stable intragastric environment [1]. However, under certain circumstances gastric acid and pepsinogen may injure the gastroduodenal mucosa. Until now, little has been known about the pathogenesis of gastric or duodenal ulcer disease [21]. However, it has been proven that inhibition of gastric acid secretion is a useful therapeutic principle for the treatment of ulcers of the upper gastrointestinal tract. Histamine-H_2-

receptor antagonists such as cimetidine, ranitidine (21a), famotidine, nizatidine [22, 23] and roxatidine [24] are clinically widely used. The use of antimuscarinic drugs for inhibition of gastric acid secretion in ulcer patients is less common and gastrin receptor antagonists (proglumide) are not potent enough to be clinically useful.

H_2-receptor antagonists are quite effective in some peptic acid disorders (duodenal ulcers, gastric ulcers), whereas their effectiveness in others is less apparent (Zollinger-Ellison syndrome, gastrooesophageal reflux disease) [22]. For such conditions, prolonged and potent reduction of acid secretion caused by H^+/K^+-ATPase inhibitors is necessary and results in superiority of omeprazole over H_2-receptor antagonists [25, 26].

BIOMEDICAL CHARACTERIZATION

IN VITRO

The effect of gastric H^+/K^+-ATPase inhibitors on enzyme activity (ATP cleavage) can be studied *in vitro* with partly purified H^+/K^+-ATPase preparations [27]. This assay has been used more effectively to study the mechanism of action of H^+/K^+-ATPase inhibitors in detail than to study the structure-activity relationship of such inhibitors [28]. Since H^+/K^+-ATPase inhibitors of the omeprazole-type need acid activation and the enzyme assay should be performed at neutral pH values, a pre-incubation period at the lowest possible pH of about 6 was used to initiate the acidic conversion of the test compound into its active principle. This reflects more the chemical instability of the test compound at neutral pH values than its effect during conditions of much higher acidity within the secretory cannaliculus of the parietal cell during acid secretion. Many chemically labile inhibitors are therefore very active in this test system. However, they do not cause an inhibition in more complex test systems and, therefore, are without any practical usefulness [28].

Proton transport studies in intact gastric vesicles, which form a pH gradient similar to *in vivo* conditions, are more suitable for studying the mechanism of action, acid-conversion and structure–activity relationship of H^+/K^+-ATPase inhibitors [29–31].

Gastric acid formation *in vitro* has been studied very intensively in isolated parietal cells from dogs [32], guinea-pigs [33] and rabbits as well as in whole gastric glands from rabbits [32] and humans [34] by means of the accumulation of the weak base ^{14}C-aminopyrine (pK_a 5.0) within the secretory compartment of the parietal cell [35]. Furthermore, oxygen

consumption correlates with acid formation [35] and has been very useful to identify artefacts of inhibition of ^{14}C-aminopyrine accumulation by neutralization of the acidic compartment by the basic nature of the test compound [30]. During artefact conditions, ^{14}C-aminopyrine accumulation is reduced by neutralization of the acidic compartment by the test compound even when the H^+/K^+-ATPase is still active, and oxygen consumption is uninhibited. The ^{14}C-aminopyrine accumulation technique has been widely used to study structure–activity relationships of gastric acid inhibitors as well as to separate the inhibitory effect of H^+/K^+-ATPase inhibitors from that of receptor antagonists. For instance, histamine-H_2-receptor antagonists inhibit only during histamine stimulation but cause no inhibitory effect during dbcAMP stimulated ^{14}C-aminopyrine uptake. In contrast, H^+/K^+-ATPase inhibitors inhibit both kinds of stimulation [27, 36].

IN VIVO

Gastric acid secretion can be studied *in vivo* in rats and dogs. Conscious rat models are rats which are pylorus-ligated [37] or which have chronic gastric fistula [38], while the rats with stomach-lumen perfusion are anaesthetized [39]. Gastric acid secretion can be studied under basal conditions as well as during stimulation of gastric acid secretion with an i.v. infusion or s.c. injection of a secretagogue: histamine, carbachol, gastrin.

From biomedical experience with different kinds of gastric acid inhibitors, the conscious dog seems to be the most relevant animal species for the prediction of the antisecretory potential of a test compound in humans. Gastric acid secretion can be studied in the dog with a chronic gastric fistula or with a Heidenhain pouch [40].

'Cytoprotection' was originally defined as the potential of a test compound in non-antisecretory doses to protect the gastric mucosa of rats against necrotizing agents such as absolute ethanol, 0.6 N hydrochloric acid, 0.2 N sodium hydroxide, 25% sodium chloride or boiling water [41]. Several prostaglandins caused 'cytoprotection', particularly in rats, in a dose-range which had no antisecretory activity. However, clinical experience with prostaglandins has shown that ulcer healing was only achieved at antisecretory doses [28]. Therefore, it seems very likely that the 'cytoprotective property' of a compound in rats has very limited relevance to predictions of its ulcer healing potential in humans if 'cytoprotection' is really separated from its antisecretory potential.

IRREVERSIBLE H^+/K^+-ATPase INHIBITORS

COMPOUNDS

Systematic synthesis of inhibitors of gastric acid secretion with a different mechanism of action from receptor antagonism started in the early 1970s at AB Haessle, Sweden [28, 42]. Starting with 2-pyridylthioacetamide (CMN 131) as a lead compound this group in 1974 synthesized timoprazole, the first well-defined inhibitor of gastric H^+/K^+-ATPase, which was followed by picoprazole in 1976 and omeprazole in 1979 (*Figure 4.4a*) [27, 42, 43]. Chemically, the basic structure consists of a substituted benzimidazole ring and a substituted pyridine ring connected to each other by a methylsulphinyl chain. Brändström *et al.* [42] claimed that these three structural elements are essential for the biological effect and that only a few compounds with ring systems closely related to benzimidazole showed weak pharmacological activities. In the meantime, other pharmaceutical compa-

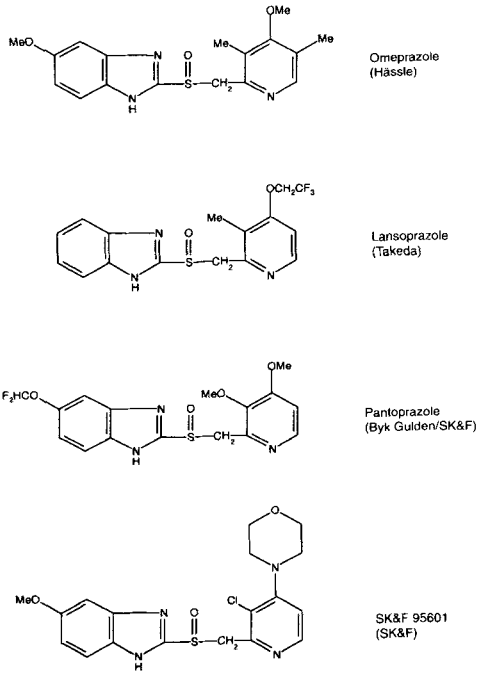

Figure 4.4a. Chemical structures of irreversible H^+/K^+-ATPase inhibitors based on the omeprazole framework currently or previously under development.

nies have described their H^+/K^+-ATPase inhibitors. Due to omeprazole's highly specific mode of action, the possibilities for structural modifications are very restricted. Indeed, most of the known omeprazole-like H^+/K^+-ATPase inhibitors are 2-[(2-pyridylmethyl)sulphinyl]benzimidazoles, differing in their substitution pattern. Only in a few analogues have the heterocyclic rings been exchanged by other moieties, for example, the pyridylmethyl group by 2-aminobenzyl group or the benzimidazole by thienoimidazole.

Lansoprazole (AG-1749, Takeda) [44], pantoprazole (BY 1023/SK&F 96022, Byk Gulden/SK&F) [45], SK&F 95601 (SK&F) [46], disuprazole (Upjohn) [47, 48], E-3810 (Eisai) [49] and Ro 18-5364 (Hoffmann-La-Roche) [50] are based on the omeprazole framework (*Figure 4.4a, b*). Saviprazole (HOE 731, Hoechst) [51] contains a thieno[3,4-d]imidazole group instead of the benzimidazole moiety of the omeprazole framework (*Figure 4.5*). NC-1300, NC-1300-B (Nippon Chemiphar) [52] and S 3337 (Hoechst) [53] are substituted benzimidazole derivatives in which the 2-pyridylmethyl moiety has been replaced by a 2-aminobenzyl group (*Figure 4.5*). BY 308 (Byk Gulden) [54] represents a sulphide based on the omeprazole framework and it has been characterized as a prodrug which needs oxidation *in vivo* to the corresponding sulphoxide.

Both enantiomers of lansoprazole inhibit with equal activity dbcAMP-

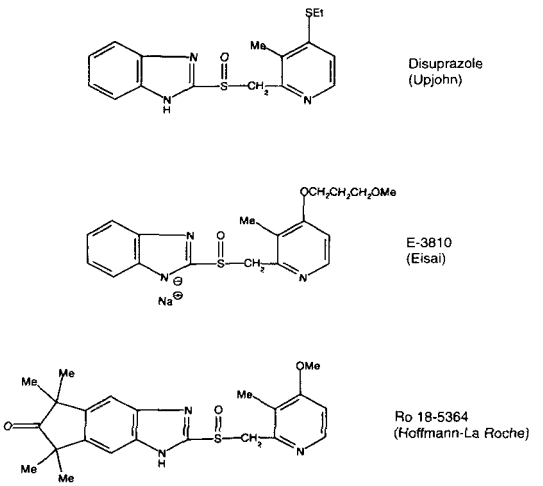

Figure 4.4b. Chemical structures of irreversible H^+/K^+-ATPase inhibitors based on the omeprazole framework currently or previously under development.

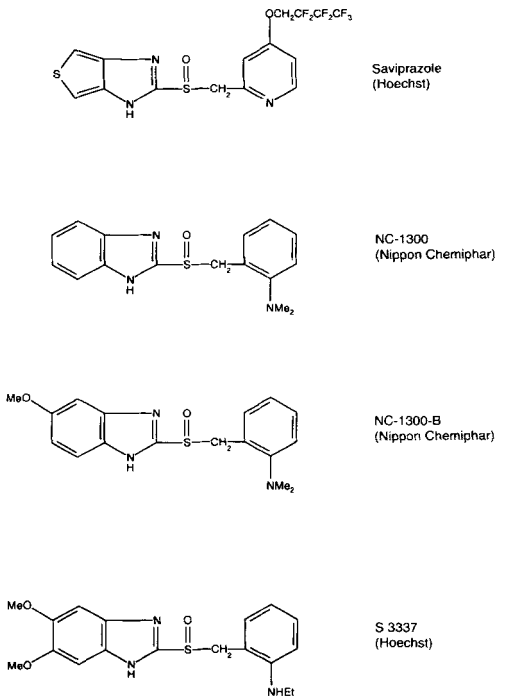

Figure 4.5. Chemical structures of irreversible H^+/K^+-ATPase inhibitors, which have modifications to the omeprazole framework, currently or previously under development.

induced aminopyrine uptake into isolated canine parietal cells as well as H^+/K^+-ATPase activity in canine gastric microsomes [55]. Similar findings were observed for the enantiomers of Ro 18-5364 [56].

MECHANISM OF ACTION

It was soon recognized that gastric H^+/K^+-ATPase is the site of action for omeprazole [27, 43] and that enzyme inhibition parallels inhibition of gastric acid secretion in laboratory animals [38]. Physico-chemically, omeprazole represents a lipid-permeable weak base with a pK_a of 4 [42]. At physiological pH, it is predominantly unionized and this neutral form passes freely across biological membranes. However, in an acidic environment with a pH below 4, it is predominantly protonated. This results in a limited permeability of the drug [28]. Due to the unique structure of the gastric

parietal cell with its acidic compartment within the secretory cannaliculi, omeprazole is trapped within these cannaliculi in its protonated form. The protonation of omeprazole starts a chemical transformation process of the omeprazole molecule within the secretory cannaliculus [57] which finally results in the formation of a sulphenamide pyridinium salt and its binding to sulphydryl-groups of H^+/K^+-ATPase (*Figure 4.6*). The sulphenamide represents the active enzyme-inhibitor [58–62]. The structural alteration of the enzyme covalently bound to the active inhibitor inactivates the catalytic function of the enzyme [28].

Two molecules of the active intermediate of omeprazole bind to one active site of gastric H^+/K^+-ATPase [63, 64]. This binding is a disulphide linkage and can be prevented and reversed by the addition of mercaptan [65–67]. Detailed investigations of three reactions of H^+/K^+-ATPase enzyme cycle have shown that the K^+-stimulated ATPase-activity, *p*-nitrophenol-phosphatase(pNPPase)-activity and formation of phosphoenzyme are also inhibited [63, 68]

In summary, omeprazole represents a prodrug which is itself inactive. It needs the acidic conditions of the parietal cell to ensure its inhibitory effect on acid secretion. To prevent acid-induced transformation within the gastric lumen and binding to superficial sulphydryl groups of the gastric mucosa, omeprazole must be administered orally in a galenic formulation that prevents acid-induced activation within the gastric juice during its passage through the stomach [69] (*Figure 4.7*). During multiple-dose studies omeprazole facilitates its own enteral absorption by its specific effect on gastric acid secretion. Under these conditions, the inhibition of gastric acid secretion and resulting neutral pH values within the gastric lumen prevent acid-induced degradation of omeprazole.

The structure–activity relationship of H^+/K^+-ATPase inhibitors of the omeprazole type is based on the balance between chemical stability at neutral pH values and acid-induced conversion into the active sulphenamide. Derivatives, which are too unstable at neutral pH, are very active in the test assay of partly purified H^+/K^+-ATPase. This assay has been performed at pH 7.4 after preincubation at pH 6 of the enzyme protein with the derivative to be tested. The high activity was therefore the result of the conversion of the derivative in solutions of neutral pH values and this does not reflect the situation of high acidity within the secretory compartment of the parietal cell [28]. The derivatives which are very unstable at neutral pH do not inhibit gastric acid secretion *in vivo* because their transformation had already occurred prior to the active principle reaching the target enzyme. Chemically very stable derivatives do not show any inhibitory effect either *in vitro* or *in vivo*.

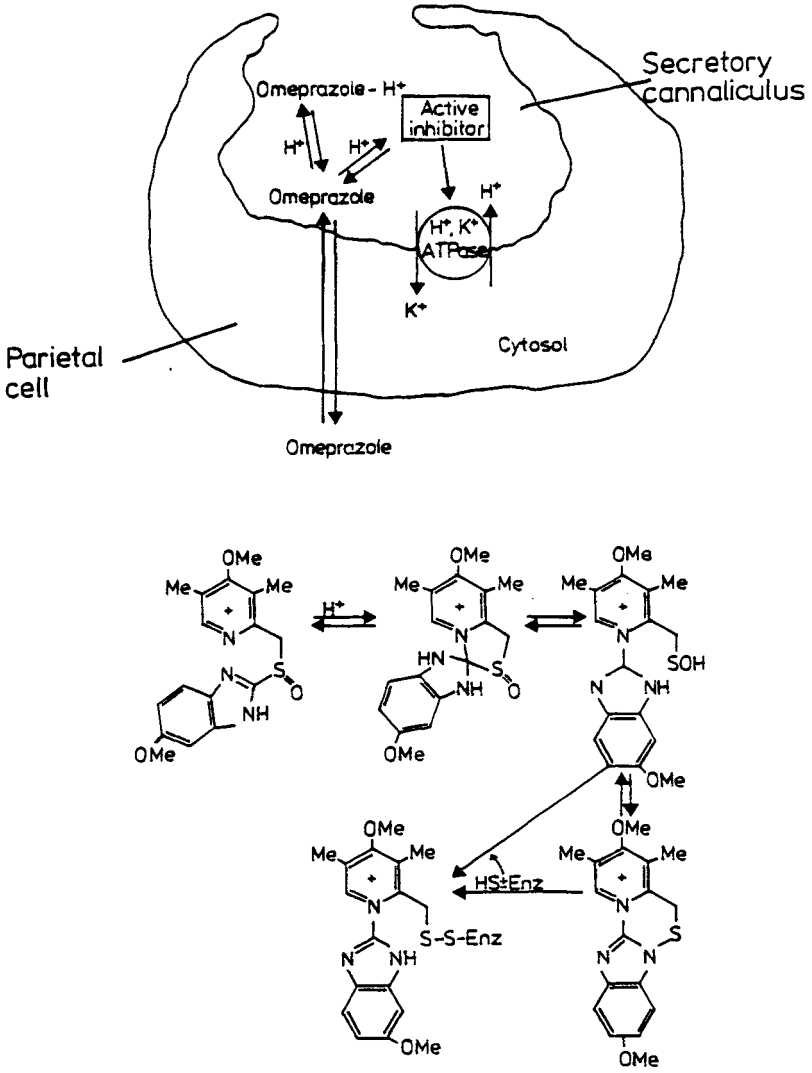

Figure 4.6. Mechanism of acid transformation of omeprazole into its active form within the secretory compartement of the parietal cell according to Lindberg et al. *[28].*

Due to the close chemical relationship between all H^+/K^+-ATPase inhibitors of the omeprazole-type (*Figures 4a,b and 5*), it seems very likely

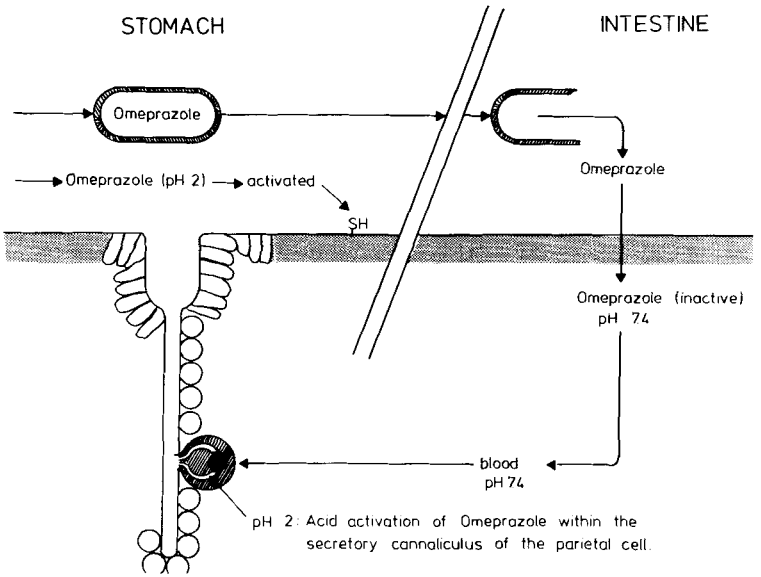

Figure 4.7. Scheme of the relation between acidic instability, intestinal absorption, and acid transformation of omeprazole-like H^+/K^+-ATPase inhibitors.

that they share the same or a very similar mechanism of action to that of omeprazole [28]. It has been reported that the sulphenamide has been identified to be the active principle for lansoprazole [70, 71], pantoprazole [45, 72] and E-3810 [73]. SK&F 95601 has been selected as a potent H^+/K^+-ATPase inhibitor with good stability at physiological pH but which undergoes acid-induced transformation to the sulphenamide as the reactive intermediate [46]. Pantoprazole demonstrates a greater chemical stability at neutral pH values than omeprazole, while its corresponding inhibitory effect on H^+/K^+-ATPase in different vesicle models was less pronounced [74]. Acid-induced transformation has also been reported for the 2-aminobenzyl substituted benzimidazoles [75, 76].

To elucidate the mechanism of action of saviprazole, chemical experiments have been performed by Scheunemann, Nimmesgern and Weidmann at Hoechst (unpublished data) (*Figure 4.8*). In the absence of mercaptans, activation induced by aqueous hydrochloric acid yields a complex mixture of products, however, with aqueous HBF_4 (50%) in methanol at −50°C the sulphenamide pyridinium salt (2) could be isolated in analogy to omeprazole [58] in high yield. This compound is highly reactive and

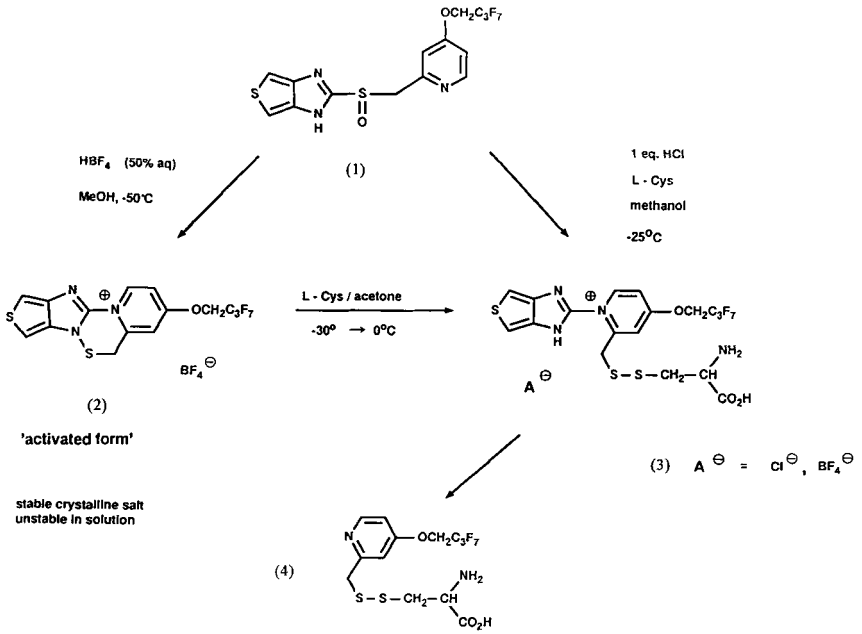

Figure 4.8. Acid activation of saviprazole to its active principle, the sulphenamide pyridium salt (2), and its L-cysteine (L-Cys) addition product (3) as a model for the enzyme–inhibitor complex.

unstable in solution. When sulphenamide (2), the active principle of saviprazole, is treated at −30°C with L-cysteine (L-Cys), which has been chosen as a model for enzyme sulphydryl-groups, the L-cysteine disulfide pyridinium salt (3) is formed. Treatment of saviprazole with L-cysteine in methanol (−25°C) in the presence of aqueous hydrochloric acid also results in the formation of the pyridinium disulphide (3). Similar experiments with omeprazole and beta-mercaptoethanol in acidic media have been reported [58, 62]. In contrast to omeprazole the saviprazole-L-cysteine adduct (3) is sensitive to cleavage into the 2-pyridyl disulfide (4) and an unidentified thienoimidazole fragment (*Figure 4.8*). The instability of the primary L-cysteine addition product (3), as a model for the enzyme inhibitor complex, may be correlated with the pharmacological profile of saviprazole, the incomplete inhibition of gastric acid secretion [51, 77].

Due to the inherent chemical instability of H^+/K^+ ATPase inhibitors of the omeprazole-type (a prerequisite for their acid-induced transformation process), there are some approaches to stabilize the substituted benzimi-

dazole molecule. Attempts to introduce appropriate prodrug moieties on the benzimidazole nitrogen which improve stability, solubility and other physicochemical properties are reported. The omeprazole-type inhibitor is then generated from these prodrugs by enzymatic or acidic cleavage [48].

ANIMAL PHARMACOLOGY

The inhibition of gastric acid secretion caused by omeprazole has been studied in rats and dogs. Omeprazole causes dose-dependent inhibitions of basal and stimulated gastric acid secretion in these species. As mentioned above, omeprazole causes an inhibition of gastric acid secretion which is independent of the kind of stimulation [38, 78]. ID_{50} values are not statistically significantly different when dose–response curves are compared either during basal secretion or during histamine-, carbachol- or gastrin-stimulated gastric acid secretion in rats [77]. Under experimental pharmacological conditions, omeprazole was less effective when administered orally as either solution or suspension in comparison with intraduodenal or intravenous administration [38, 79]. This is in accordance with its pH-dependent conversion which occurs to a significant proportion within the gastric juice under experimental conditions after oral administration, preventing the active principle derived from acid-induced transformation reaching the target enzyme at the apical membrane of the parietal cell (*Figure 4.7*).

Similar findings have been reported for lansoprazole [80, 81] and saviprazole [77]. Substituted thienoimidazoles such as S 1924 and saviprazole show, in doses producing more than 90% inhibition, a more pronounced residual secretion rate than omeprazole after a single administration to rats and dogs [53, 77]. Despite its significantly lower potency compared with omeprazole in Heidenhain-pouch dogs, SK&F 95601 has been selected for clinical trials because of its higher chemical stability at physiological pH [46]. It has been shown that E-3810 is twice as potent as omeprazole in dogs [82]. Compounds, in which the 2-pyridylmethyl group is replaced by a 2-aminobenzyl moiety, for example, NC-1300, NC-1300-B [52, 83] and S 3337 [53], inhibit gastric H^+/K^+-ATPase and gastric acid secretion in pylorus-ligated rats at the same concentration and dose range as omeprazole. Furthermore, the duration of action for NC-1300-B was much longer than that of omeprazole. NC-1300-B inhibits gastric acid secretion in conscious pylorus-ligated rats after a single pretreatment of 72 hours [52]. In contrast to the omeprazole-like potency *in vitro* and in pylorus-ligated rats S 3337 [53], NC-1300 [84] and compounds with substitutions on the nitrogen atom of the 2-

aminobenzyl moiety [76] show a significantly lower potency than omeprazole on gastric acid secretion in dogs. Recently, attempts have been reported to replace the benzimidazole by an imidazole moiety in connection with an N-substituted 2-aminobenzyl instead of the pyridine ring, resulting in effective H^+/K^+-ATPase inhibitors with good antisecretory potencies even in dogs [84].

Gastric acid inhibition lasts 3–4 days in dogs after a single administration of omeprazole of a dose which causes a maximal inhibition at the day of administration [38, 79]. This long-lasting inhibition is in contrast to clinically available H_2-receptor antagonists which inhibit histamine-induced gastric acid secretion for only 4 to 18 hours. This long duration of acid inhibition caused by omeprazole results from the irreversible covalent binding of the acid-induced sulphenamide to sulphydryl groups of the H^+/K^+-ATPase. Gastric acid secretion reappears only when new enzyme is synthesized. The half-life of the gastric H^+/K^+-ATPase in rats is about 48 hours [85]. Therefore, acid secretion inhibited by omeprazole reappears within 24 hours and returns to normal in about 3–4 days [3]. Since there is a significant residual inhibition after 24 hours, the degree of inhibition increases during the first days of repeated daily administration. A constant inhibitory level is achieved after about four days of treatment [86].

In contrast to omeprazole it has been claimed that for lansoprazole *de novo* synthesis of the H^+/K^+-ATPase does not participate in the process of recovery from inhibition but that gluthathione is somehow involved in the reactivation of the enzyme [71]. For saviprazole, it has also been suggested that the fading inhibitory profile, which led to the more pronounced residual secretion rate [77, 87], is caused by perturbations of the cellular glutathione level [31]. The duration of action of E-3810 in dogs is significantly shorter compared with omeprazole [82]; it is suggested that endogenous extracellular glutathione is involved in the reactivation process *in vivo* [88].

As H^+/K^+-ATPase inhibitors of the omeprazole-type need acid for their conversion into their corresponding active principle, any condition in which gastric acid secretion is inhibited results in reduced drug-induced inhibition. For example, if gastric acid secretion is inhibited by a short-acting H_2-receptor antagonist and omeprazole is administered during conditions of maximal-inhibited acid formation at a dose which causes a 3–4 day inhibition, the omeprazole-induced inhibition will be significantly reduced. The inhibitory effect of omeprazole depends on the secretory state of the parietal cell [67, 89]. Similar findings have been reported for pantoprazole [90].

In addition to the antisecretory effect, it has been reported that omeprazole has cytoprotective properties in rats which are not mediated by

its inhibitory effect on gastric acid secretion [91, 92]. For pantoprazole it has been suggested that the anti-ulcer property is somehow related to its antisecretory effect [93]. For lansoprazole, it has been claimed that the anti-ulcer effect in rats is 3 to 10 times more pronounced than with omeprazole [80]. However, from the clinical experience with omeprazole, there is no doubt that omeprazole exerts its effect on ulcer healing in humans predominantly via its antisecretory activity [20, 94]. This fact is confirmed by the clinical experiences with omeprazole and lansoprazole: both compounds are equally effective in ulcer healing in humans at similar doses [20, 94, 95] to those which express their antisecretory effect, although lansoprazole is 3 to 10 times more potent in rat ulcer models. Therefore, it may be justifiably concluded that the rat ulcer models have only a very minor relevance for ulcer healing in humans.

HUMAN PHARMACOLOGY

Human pharmacology parallels animal pharmacology, especially that of the dog, with respect to the effective dose-range and duration of action. Omeprazole causes dose-dependent inhibition of basal and stimulated gastric acid secretion and reduction of intragastric acidity in healthy volunteers after single administration [96–98] or repeated daily administrations [96]. This was also seen in patients with peptic ulcer disease [99–102]. The inhibitory effect after a single administration lasts 2–3 days [96].

Lansoprazole causes dose-dependent inhibition (15, 30, 60 mg) of stimulated and basal gastric acid secretion in healthy volunteers. It increases intragastric pH and has a long duration of action [103, 104]. Morning dosing for lansoprazole is recommended due to circadian differences in bioavailability [105]. The administration of 40 or 60 mg pantoprazole for five days maintained gastric pH above 3 for 33% or 58% of time, respectively, compared with 15% of time after placebo [106]. In another study, 40 mg pantoprazole for five days increased gastric pH to about 4 [107]. A single intravenous dose SK&F 95601 caused dose-dependent inhibition of pentagastrin-stimulated gastric acid output in healthy volunteers at doses between 16 and 96 mg [108].

PHARMACOKINETICS

After administration of omeprazole intraduodenally to dogs or buffered suspensions containing omeprazole perorally to humans, omeprazole is rapidly absorbed from the gut; peak plasma levels are reached within 30 minutes [38, 96]. After administration in an enteric-coated formulation,

intestinal absorption of omeprazole is delayed and plasma concentration-time curves are flat and broad with low peak plasma concentrations [69]. Subsequently, omeprazole is eliminated from the plasma with a half-life of 40–60 minutes, depending on the species [38, 96]. There is no correlation between the duration of the inhibitory effect caused by omeprazole (up to 4 days) and its plasma concentration at a given time ($t_{1/2}$ less than 60 minutes). However, the area under the plasma concentration–time curve (AUC) during 0–4 hours correlates well with the inhibitory effect both in dogs [38] and humans [96]. Therefore, the shape of the plasma concentration-time curve is of no importance and different formulations resulting in equal AUCs will cause equal inhibitory effects [69]. This indicates that the amount of omeprazole being absorbed from the gut correlates with the amount of omeprazole being transported by the blood to the parietal cell and being available for inhibition of the H^+/K^+-ATPase [28].

Lansoprazole, administered to healthy volunteers as encapsulated enteric-coated granules, disappeared from the blood with a half-life ranging between 1 and 2 hours [95].

In this context, the results of autoradiographic studies in mice with ^{14}C-omeprazole are very impressive. The radioactivity was present only in the gastric wall 16 hours after intravenous injection of ^{14}C-omeprazole [109]. This indicates that the active enzyme inhibitor is present for many hours only at the site of its action. This correlates closely with its long-lasting duration of action while the parent compound has rapidly disappeared from the blood. Similar results were obtained for saviprazole (Eckert, personal communication).

METABOLISM

Omeprazole is rapidly and completely metabolized after intestinal absorption. Oxidative processes for metabolism are predominant and three main metabolites of omeprazole have been identified: the corresponding sulphone and sulphide as well as hydroxyomeprazole. Omeprazole sulphone is further metabolized or it is eliminated in the faeces. Renal excretion is the predominant route of elimination of omeprazole metabolites [20, 28, 110]. The metabolism of lansoprazole in humans is comparable to that of omeprazole; it is converted to hydroxylansoprazole, lansoprazole-sulphone, lansoprazole-sulphide and the hydroxylated sulphone [111].

In hepatic microsomes, omeprazole inhibited cytochrome P450-mediated metabolic reactions *in vitro*. This effect was comparable to the inhibition caused by cimetidine with respect to the extent of the inhibitory effect and the effective concentrations [110]. Under well-defined clinical conditions, an

interaction in drug metabolism has been detected between omeprazole and diazepam, phenytoin [112] or warfarin [113]. However, clinically significant drug interactions appear to be unlikely [114]. It has been reported for pantoprazole that the interaction with cytochrome P450 *in vitro* [115] and *in vivo* [116] is less than with omeprazole and lansoprazole while its overall antisecretory potency and efficacy is similar to that of omeprazole [117]. Additionally, pantoprazole does not influence the disposition kinetics of theophylline in man [118].

ENTEROCHROMAFFIN-LIKE CELL PROLIFERATION

Omeprazole was well tolerated in chronic toxicological studies in rats or dogs up to the highest tested oral doses of 414 mg/kg or 138 mg/kg, respectively [28]. However, after 2 years of treatment (cancerogenicity study in rats), a dose-dependent increase in gastric carcinoids were more pronounced in female than in male rats [86, 119]. Carcinoids in rats consist of enterochromaffin-like cells (ECL-cells) which are predominantly formed by histamine-releasing cells in the rat gastric mucosa [120] and differ, therefore, from carcinoids in humans which are normally formed by serotonin-producing cells [86]. Similar results have been reported for long-acting H_2-receptor antagonists: BL-6341 [121], loxtidine [122, 123] and SK&F 93479 [124]. These findings are more likely to be due to the common pharmacodynamic effect of these H_2-receptor antagonists and omeprazole causing a long-term inhibition of gastric acid secretion than an inherent carcinogenic effect of these different compounds [125].

There were initial doubts [126–128] concerning attempts to explain the occurrence of carcinoids in rats by the drug-induced excessive hypergastrinaemia. From the present point of view, these initial doubts have not been proven to be valid because subsequent results which support the gastrin hypothesis [125, 129] are convincing.

ECL-cells are functionally and trophically under the control of gastrin [120]. The release of gastrin, synthesized in antral G-cells, is mediated by the amino acid composition of the gastric juice as well as by its acidity. Acidic pH values inhibit antral gastrin release. Neutral pH values do not stimulate gastrin release but represent the removal of the natural break for gastrin release [3]. Any pharmacologically-induced inhibition of gastric acid secretion, therefore, must finally lead to increased antral G-cell density with corresponding increased serum gastrin levels and ECL-cell proliferation. This has been demonstrated for omeprazole [130–134], lansoprazole [135], saviprazole (Engelbart, personal communication), ranitidine [131–134] and even for antacids [136, 137]. Even different degrees of surgical removal of

acid-secreting mucosa (75, 90, 100% fundectomy) in rats without any administration of gastric acid inhibitors resulted finally in a stepwise increase in serum gastrin indicating that the antral acid load (or pH) appears to be the major factor for gastrin release [138]. In addition, antrectomy, the removal of the gastrin-releasing mucosa, prevented ECL-cell proliferation in omeprazole-treated rats [139], although antrectomy did not accelerate reversal of omeprazole-induced ECL-cell hyperplasia [140]. The long-lasting hypergastrinaemia during life-long treatment with omeprazole in rats finally induced carcinoids. Therefore, the occurrence of carcinoids in the cancerogenicity study in rats was not caused by an inherent carcinogenic effect of omeprazole but was pharmacodynamically induced by its long-lasting effect of gastric acid inhibition with subsequent excessive hypergastrinaemia [125, 129].

In humans, omeprazole treatment as well as treatment with H_2-receptor antagonists cause a moderate increase in serum gastrin which reflect the degree of gastric acid inhibition [141]. There were no detectable omeprazole-induced pathological changes in the gastric mucosa in patients with peptic ulcer or reflux oesophagitis in whom therapy was extended for 1–4 years [142, 143]. From the clinical experience of chronic gastritis progressing over years, long-lasting achlorhydria is accompanied by a slight hyperplasia but no dysplasia of the ECL-cells in the gastric mucosa. This observation parallels the findings after omeprazole treatment up to 5 years; it is recommended that plasma gastrin levels be monitored in patients receiving long-term treatment [144] and the dose of omeprazole should be reduced if plasma gastrin values increase above 5 times the upper limit of normal [145].

THYROID TOXICITY

It has been reported that high doses (138 and 430 mg/kg) of omeprazole to rats interfere with the peripheral conversion of thyroxine (T4) to tri-iodothyronine (T3) resulting in the decrease of serum T3, unchanged serum T4 and no change in the morphology of the thyroid gland [119].

We studied the effect of omeprazole, lansoprazole, saviprazole, SK&F 65601 and E-3810 on total serum T4 and T3, free serum T4 and T3 as well as on thyroid weights after oral treatment for 14 days at doses of 50, 150 and 450 mg/kg [146]. The selected doses represent supra-maximal doses with respect to their antisecretory effects in rats (ED_{50} in pylorus-ligated rats: 1–5 mg/kg intraduodenally).

Free serum T4 and T3 values parallel total serum T4 and T3. Omeprazole and SK&F 65601 caused no changes of the thyroid weights and slightly elevated serum T4 and T3 values without showing dose-dependencies

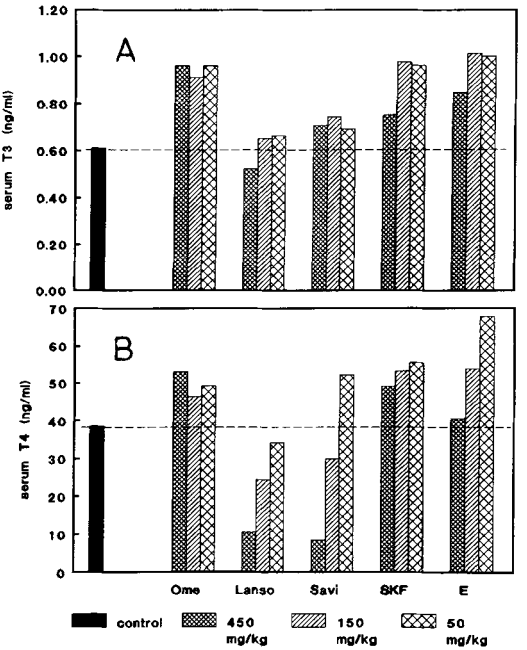

Figure 4.9. Total serum T3 (A) and total serum T4 (B) in rats after treatment with omeprazole, lansoprazole, saviprazole, SK&F 95601 and E-3810 at doses of 50, 150 and 450 mg/kg orally for 14 days. Values are means +/− S.E.M., n=9–10 rats.

(*Figure 4.9*). The treatment period of 14 days was too short to expect the reported decrease in total serum T3 for omeprazole, which was obvious at the end of the 3 month toxicity study [119]. E-3810 caused significant elevated serum T4 and T3 values with an inverse dose-dependency (*Figure 4.9*) without changes of the thyroid weights. Saviprazole and lansoprazole dose-dependently decreased total serum T4 without affecting serum T3 levels (*Figure 4.9*). Although both compounds showed the same degree of lowering serum T4, there was a significant weight increase of the thyroid gland only after the highest dose of saviprazole. The highest dose of lansoprazole showed a comparable tendency to increase thyroid weight after the treatment period was prolonged to 6 weeks.

Further studies with saviprazole to elucidate the mechanism of the decrease in serum T4 revealed that the compound does not interfere with hormone synthesis, storage or release from the thyroid gland. The decrease in peripheral T4 was caused by an increased hepatobiliary elimination of T4

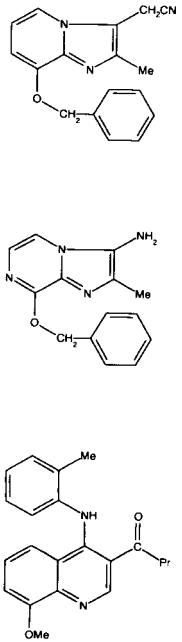

Figure 4.10. Chemical structures of reversible H^+/K^+-ATPase inhibitors currently or previously under development.

due to induction of hepatic T4-metabolizing enzymes. The increase in thyroid weight induced by 450 mg/kg saviprazole in rats could be dose-dependently prevented by the exogenous substitution of T4.

REVERSIBLE H^+/K^+-ATPase INHIBITORS

COMPOUNDS

SCH 28080 (Schering-Plough) [147] is the prototype of a reversible H^+/K^+-ATPase inhibitor (*Figure 4.10*). It was soon recognized that SCH 28080 has antisecretory properties in various *in vitro* and *in vivo* assays [148] and cytoprotective properties in different rat ulcer models [149]. As early as 1983 it was suggested that the antisecretory effect might involve gastric H^+/K^+-ATPase [148], although during the subsequent years the predominant scientific interest concentrated on its cytoprotective potential. The

clinical development of SCH 28080 was discontinued because of liver toxicity in animals and elevated liver enzyme activity in serum of human volunteers [150]. The follow-up compound SCH 32651 (Schering-Plough [151] (*Figure 4.10*) was 4 times more potent in causing cytoprotective effects in rats [152] compared with its antisecretory potential [153]. During characterization of its antisecretory effect in guinea-pig isolated fundic mucosa, it was also concluded that SCH 32651 seems to act directly on the parietal cell, at or near the site of H^+/K^+-ATPase [154].

Systematic synthesis was carried out by Smith Kline & French to identify freely reversible, noncovalent inhibitors of gastric H^+/K^+-ATPase with a mode of action comparable with SCH 28080. It was expected that gastric H^+/K^+-ATPase inhibitors with a shorter duration of action could be of therapeutic interest [155, 156]. This research resulted in the selection of compound SK&F 96067 [157] (*Figure 4.10*).

MECHANISM OF ACTION

The assumption that SCH 28080 and SCH 32651 cause their antisecretory effect by direct interference of gastric H^+/K^+-ATPase was confirmed in 1985 [158]. SCH 28080 inhibits the enzyme activity in a concentration dependent manner with an IC_{50} in the micromolar range comparable to that of omeprazole. The effect of SCH 32651 was obviously less pronounced [158, 159].

The difference between omeprazole and SCH 28080 in their ability to inhibit gastric H^+/K^+-ATPase is dependent on their inhibition kinetics. In contrast to omeprazole, SCH 28080 competes with the high affinity K^+-site on the gastric H^+/K^+-ATPase. Its effect on Na^+/K^+-ATPase activity is much less pronounced in comparison with its effect on gastric H^+/K^+-ATPase activity [159, 160]. SCH 28080 is a protonatable weak base ($pK_a = 5.6$) which accumulates in acidic compartments in the same way as omeprazole on the lumenal, acidic side of the parietal cell membrane in a protonated form [161]. However, SCH 28080 is chemically stable and active by itself after protonation [162] and does not need an acid-induced transformation such as required by omeprazole-like irreversible inhibitors. Therefore, in proton transport studies, SCH 28080 inhibits the initial rate of H^+/K^+-ATPase mediated H^+accumulation and the steady state proton concentration. This is in contrast to omeprazole, which first needs accumulation of acid within gastric vesicles to generate an interior of low pH to facilitate the acid-induced transformation prior to being able to inhibit the H^+/K^+-ATPase [163]. SCH 28080 binds to the lumenal side of H^+/K^+-ATPase [161,

164]. Its inhibitory effect can be reversed by dilution or washing and cannot be prevented by the addition of sulphydryl reducing agents [165].

SK&F 96067 also binds competitively to the lumenal K^+-binding site of gastric H^+/K^+-ATPase and is active in its protonated form [166].

PHARMACOLOGY

According to the mechanism of action of SCH 28080, inhibition of gastric acid secretion is independent of the kind of stimulation. In guinea-pig isolated fundic mucosa, SCH 28080 inhibits histamine-, methacholine- or dbcAMP-stimulated acid secretion concentration-dependently with IC_{50} values in the micromolar range [154]. Similar results have been obtained *in vivo*. Histamine-, dimaprit- and pentagastrin-induced gastric acid secretions in dogs are inhibited by similar doses [149]. The antisecretory effect of SCH 28080 in human volunteers was first demonstrated in 1982 [167], before its mechanism of action had been elucidated.

SK&F 96067 inhibits gastric acid secretion dose-dependently in rats and dogs. Its duration of action is longer than that of cimetidine but much shorter than that of omeprazole [157, 168].

OTHER INHIBITORS

The inhibitory effects of verapamil, a calcium channel antagonist, on gastric acid secretion *in vitro* and *in vivo* are controversial. There are reports that verapamil causes a significant inhibition of acid secretion in humans [169], and rats [170, 171] but others claim that it has no effect [172, 173].

It is generally accepted that the interference with acid formation in gastric parietal cells caused by verapamil is not due to its calcium channel antagonism [174]. As early as 1983, it was reported that verapamil and gallopamil inhibit gastric acid formation in isolated parietal cells. The inhibition was independent of the kind of stimulation (histamine, dbcAMP and K^+). From these results, it was concluded that verapamil and gallopamil inhibit gastric acid secretion by interfering with the parietal cell proton pump [175]. Even early kinetic studies indicated that the inhibitory effect of verapamil on H^+/K^+-ATPase is of a competitive manner with respect to K^+ [176]. Similar findings were obtained with trifluoperazine, an antipsychotic drug, and TMB-8, an antiarrhythmic agent [176]. However, in rabbit isolated gastric glands verapamil has been shown to have non-specific inhibitory effects on acid formation with various IC_{50} values depending on the agonist used [177]. These nonspecific inhibitory effects have been characterized as being of a protonophoric [178] and detergent [179] nature.

The interference of some neuroleptics and antidepressants such as trifluoperazine, doxepin and trimipramine with gastric H^+/K^+-ATPase is caused by an allosteric mechanism at the K^+-site of the enzyme [163, 180].

It has recently been suggested that stimulation of gastric acid secretion across the apical membrane of the gastric parietal cell predominantly reflects the insertion of an active potassium and chloride transporter/channel rather than direct activation of H^+/K^+-ATPase. However, the mechanisms involved in K^+ and Cl^- transport into the gastric parietal cell are still controversial. The presence of a Cl^- channel in the apical membrane has been confirmed by using established Cl^- channel blockers. *In vitro* in rabbit isolated gastric parietal cells, 9-anthracene carboxylate and the more potent diphenylamine-2-carboxylate inhibit gastric acid formation concentration-dependently irrespective of the kind of stimulation [181]. Thus Cl^- channel blockers can be defined as indirect H^+/K^+-ATPase inhibitors.

CONCLUSIONS

The superiority of omeprazole-like irreversible H^+/K^+-ATPase inhibitors over H_2-receptor antagonists in acid-related disorders of the upper gastrointestinal tract is quite obvious. Their clinically relevant advantages are related to their different mode of action which is independent of the kind of acid stimulation and to their longer duration of action. Reversible H^+/K^+-ATPase inhibitors share the secretagogue independent mode of action with the irreversible inhibitors but show a duration of action which is comparable with that of H_2-receptor antagonists. Their clinical advantage over irreversible inhibitors has still to be proven. Other H^+/K^+-ATPase inhibitors such as Cl^- channel blockers are, at present, of experimental interest only.

ACKNOWLEDGEMENT

The authors wish to thank Dr. P. Godden for revising the manuscript.

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Progress in Medicinal Chemistry – Vol. 31, edited by G.P. Ellis and D.K. Luscombe

5 Semi-Synthetic Derivatives of 16-Membered Macrolide Antibiotics

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INTRODUCTION

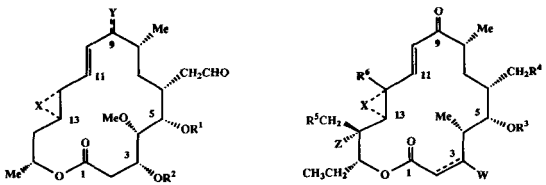
Macrolides are a large and diverse class of natural products isolated from the culture broths of certain soil micro-organisms [1–3]. Several members of this class have been commercially developed as antibiotics to treat susceptible pathogens in clinical and/or veterinary medicine [4, 5]. These fermentation-derived compounds have also been extensively utilized as starting materials for the synthesis of many derivatives by both chemical and biochemical methods [6]. Several useful semi-synthetic derivatives which have arisen from these efforts have contributed substantially to the current renaissance that the macrolide antibiotics are enjoying [2, 7–11].

The fermentation-derived macrolides are classified according to the ring size and substitution pattern of their aglycones, that is, their core lactone ring systems lacking the amino and/or neutral sugars usually present in the complete molecular structure. The two predominant families possess either a 14- or a 16-membered aglycone [1–3]. Semi-synthetic derivatives of erythromycin, a 14-membered macrolide and the most widely used macrolide antibiotic, were recently reviewed [12]. In this chapter, the semi-synthetic 16-membered macrolides are reviewed.

CLASSIFICATION OF 16-MEMBERED MACROLIDES

The 16-membered macrolide antibiotics are generally divided into two principal families, the leucomycin-related family and the tylosin-related family, on the basis of the substitution pattern of their aglycones [1–3]. Generalized structures for these two types of aglycones are illustrated in *Figure 5.1.*

Leucomycin-related macrolides possess the representative aglycone (1a) (*Figure 5.1*). The most common variations of functionality include: X at



1a 1b

Figure 5.1. Comparative substitution pattern of aglycones for leucomycin-related (1a) *and tylosin-related* (1b) *16-membered macrolides.*

C-12,13 is a double bond or β-epoxy group; Y at C-9 is a ketone or α-hydroxyl group; R^1 is the disaccharide, 4′-*O*-(α-L-mycarosyl)-β-D-mycaminosyl, in which the 4″-hydroxyl group of the mycarosyl subunit is usually acylated by a short-chain fatty acid; and R^2 is hydrogen or a short-chain acyl group. As a consequence of the many possible permutations and combinations of substitution patterns, the leucomycin-related macrolides are often produced as multi-factored complexes from which the individual components are separated. The production of related mixtures by different organisms has yielded both a wide diversity of structural variations and the isolation of identical compounds from different cultures. As a result of this latter rather common feature, many individual compounds have acquired different names or numerical designations.

The spiramycin family is often classified separately from the leucomycin family despite the close resemblance of their respective aglycones. The spiramycins are structurally distinct in that the 9-α-hydroxyl group of their aglycone is glycosylated by a β-D-forosaminyl moiety. Having this second amino sugar incorporated into the typical macrolide structure, the spiramycins are unique dibasic macrolides.

Aglycones of the second major division within 16-membered macrolides, the tylosin-related family, exhibit greater structural diversity. In structure (1b), (*Figure 5.1*) variations include: X at C-12,13 is a double bond or β-epoxy group; W at C-3 is an α-hydroxyl group when C-2,3 is a single bond or is hydrogen when C-2,3 is a double bond; Z at C-14 is hydrogen or hydroxyl; R^3 is an amino sugar which may be 4′-*O*-glycosylated; R^4 is a formyl or hydroxymethylene group or hydrogen; R^5 is hydrogen, a hydroxyl group, or an *O*-glycosyl moiety; and R^6 is methyl or hydrogen. Although members of this family have also been independently isolated from culture broths of different organisms and have thereby acquired different names or numbers, this situation has not occurred as frequently as in the leucomycin family.

The wide array of 16-membered macrolides has amply supplied chemists with starting materials for structural modifications, and the extensive structural variations have provided a wealth of ideas from which hybrid molecules have been conceived. In addition to synthesis by purely chemical means, structural modifications have been effected by bioconversions, alterations of biosynthetic pathways, and genetic manipulations of macrolide-producing micro-organisms [13]. These natural structural variations have also contributed to a better understanding of structure-activity relationships and have been used to successfully guide synthetic programmes. Finally, these complex natural products have inspired numerous

total syntheses and the development of new synthetic methods and strategies for their assembly [14–17].

MACROLIDES IN THE LEUCOMYCIN-RELATED FAMILY

LEUCOMYCIN COMPLEX AND JOSAMYCIN

The leucomycin complex is comprised of ten factors which differ in the number and type of short-chain acyl substituents on the 3- and 4″-hydroxyl groups [1–3]. In the generalized structure depicted in *Figure 5.2*, R^1 is acetyl or hydrogen, and R^2 is 3-methylbutanoyl (isovaleryl, iVal), n-butyryl, propionyl, acetyl, or hydrogen. The leucomycin complex (also called kitasamycin) was isolated from culture broths of *Streptomyces kitasatoensis* [18], an organism later reclassified into the genus *Streptoverticillium* [19].

Josamycin (2) was initially reported as a new macrolide from culture broths of *Streptomyces narbonensis* var. *josamyceticus* [20], but it was later proven to be identical to leucomycin A_3 (*Figure 5.2*, R^1 = acetyl, R^2 = isovaleryl) [21]. It was commercially developed under the name josamycin and launched initially in Japan in 1970 and subsequently in many other countries [5]. Josamycin has attained wide clinical utility and is often used as a reference standard against which new antibiotics are compared.

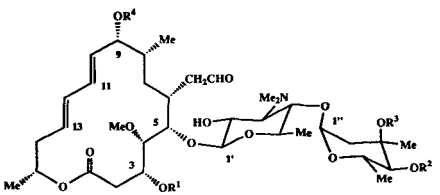


Figure 5.2. Structural variations within the leucomycin-related family of macrolides.

Table 5.1

	R^1	R^2	R^3	R^4
Josamycin (2)	Ac	iVal	H	H
Midecamycin A_1 (3)	EtCO	EtCO	H	H
Miokamycin (4)	EtCO	EtCO	Ac	Ac
Leucomycin A_5 (5)	H	PrCO	H	H
Rokitamycin (6)	H	PrCO	EtCO	H

MIOKAMYCIN

Miokamycin (4) is a semi-synthetic derivative of midecamycin A_1 (3), one of the individual factors in the complex produced by *Streptomyces mycarofaciens* [22, 23]. Miokamycin (also spelled miocamycin or called midecamycin acetate, MOM, or ponsinomycin) is 9,3″-di-*O*-acetylmidecamycin A_1 (*Figure 5.2*) [24, 25].

Extensive early studies of *in vitro* and *in vivo* structure-activity relationships within the leucomycin family revealed correlations between the number and type of *O*-acyl substituents and the compounds' antibacterial potency, efficacy in treating experimental infections, and serum antibiotic concentrations [26]. Consequently, esterification of all hydroxyl groups within several leucomycin-related macrolides was conducted to find derivatives with better antibiotic activity and pharmaceutical properties (such as greater water solubility and masking their extremely bitter taste). From such investigations with midecamycin, miokamycin was synthesized and characterized as a useful new macrolide antibiotic [24, 27]. It has now been commercially launched in several countries [5].

Due to the facile intramolecular migration of acyl groups between the 3″ and 4″-hydroxyl groups, miokamycin was originally synthesized by an indirect route. Microbial hydrolysis of the 4″-*O*-propionyl group of midecamycin was followed by 9, 2′, 4″-tri-*O*-acetylation; heating this intermediate with propionic anhydride in pyridine effected both the 4″-, to 3″-transacetylation and then the return of the 4″-*O*-propionyl group [24]. A direct synthetic route for 3″-*O*-acylation without acyl migration was later developed [25, 28]. Further investigations of this chemistry yielded additional *O*-acyl derivatives of midecamycin [29–31].

ROKITAMYCIN

Rokitamycin (6) is a semi-synthetic derivative of leucomycin A_5 (5), another of the individual factors found in the leucomycin complex along with josamycin (leucomycin A_3). Initially referred to as TMS-19-Q, rokitamycin is the 3″-*O*-propionyl derivative of leucomycin A_5 (*Figure 5.2*) [32, 33].

The lines of research that led in one direction to miokamycin [24, 26] were extended in another direction by other investigators who synthesized additional acyl derivatives of the leucomycin factors, including mono-esters of the hindered 3″-hydroxyl group. These latter derivatives were prepared via a multi-step protection (2′-*O*-acetyl; 3,9-bis-*O*-trimethylsilyl), 3″-*O*-acylation, and deprotection sequence [32]. From this series, 3″-*O*-propionylleucomycin A_5 was chosen for further development because of its superior

in vitro activity and high serum concentrations in animals [34]. Rokitamycin has recently been launched in Japan and Italy [5].

OTHER DERIVATIVES

Another macrolide ester that has received some clinical attention is 9-*O*-propionylmaridomycin (8) (*Figure 5.3*) [35, 36]. It is a semi-synthetic derivative of maridomycin III (7) [37], the major component of a complex produced by *Streptomyces hygroscopicus* [38]. 9-*O*-Propionylmaridomycin was selected as the best candidate from a series of ester derivatives that had been synthesized from the maridomycins by standard acylation methods [39, 40]. Propionyl esters of josamycin have also undergone limited clinical investigations [41–43]. Other investigators have explored the effects of esterification on the deltamycins, a complex isolated from culture broths of *Streptomyces halstedii* subsp. *deltae* [44]. Since the facile *in vivo* hydrolysis of their natural 4″-*O*-acyl substituents resulted in diminished antibacterial activity, more stable esters of deltamycin were sought. A series of 4″-*O*-phenylacetyl derivatives (10) (*Figure 5.3*), synthesized from 4″-deacyldeltamycin (9), showed modest improvements in activity and some correlations between activity and physical chemical parameters [45, 46]. However, further developments with any of these esters have not been reported recently. In addition to chemical modifications, acylations and

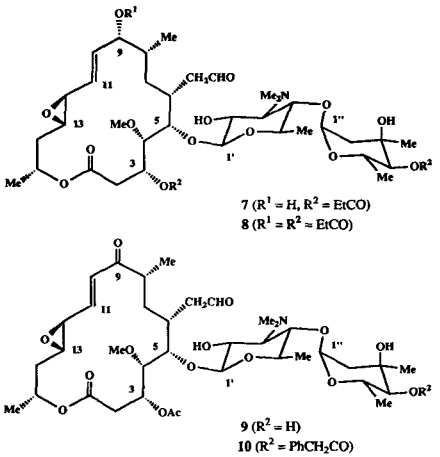


Figure 5.3. Structures of maridomycin III (7), *9-O-propionylmaridomycin* (8), *4″-O-deacyldeltamycin* (9), *and 4″-O-phenylacetyldeltamycin* (10).

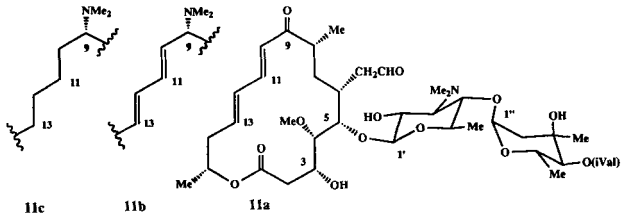


Figure 5.4. Structures of niddamycin (11a), *9-β-N,N-dimethylamino-9-deoxoniddamycin* (11b), *and 9-β-N,N-dimethylamino-9-deoxo-10,11,12,13-tetrahydro-niddamycin* (11c).

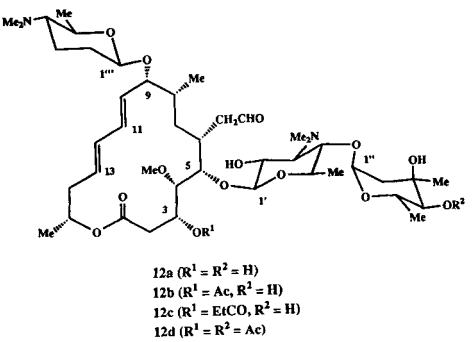
deacylations of 16-membered macrolides have been performed by microbial transformations [47, 48].

Modifications other than esterifications have been less extensively reported, although many derivatives have been described in the older literature that were prepared during structure elucidation and basic chemical studies of new macrolides. Some of this work has been compiled in reviews [1, 3, 6, 49]. None of these older compounds has been commercially developed. Results from these early studies indicated that antibacterial activity was only minimally affected by epimerization of the C-9 substituent, alkylation of the 9-hydroxyl group, saturation of the C-10,11,12,13-diene, or epoxidation of the C-12,13 double bond [26, 49–52]. The aglycone of niddamycin (11a) (*Figure 5.4*) was recently transformed into a series of 9-α-and 9-β-*N,N*-dialkylamino-9-deoxo-10,11,12,13-tetrahydro derivatives; the 9-α-*N,N*-dimethylamino analogue (A-65352) (11c) exhibited activity comparable to josamycin *in vitro* and *in vivo* [53]. The 9-α-*N,N*-dimethylamino derivative of niddamycin itself (A-75835) (11b) was subsequently reported to be more active than josamycin [54].

MACROLIDES IN THE SPIRAMYCIN FAMILY

The spiramycin complex, initially isolated from culture broths of *Streptomyces ambofaciens*, contains three major factors which possess the same aglycone as the leucomycins, but differ from each other by their 3-*O*-acyl substituent (12a)–(12c) (*Figure 5.5*) [55]. The spiramycins are uniquely distinguished from other 16-membered macrolides by their 9-*O*-β-D-forosaminyl substituent [56, 57]. A monograph devoted to more recent evaluations of spiramycin has been published [58].

From the numerous esters of spiramycin that were initially prepared,



12a ($R^1 = R^2 = H$)
12b ($R^1 = Ac, R^2 = H$)
12c ($R^1 = EtCO, R^2 = H$)
12d ($R^1 = R^2 = Ac$)

Figure 5.5. Structures of spiramycin I (12a), *spiramycin II* (12b), *spiramycin III* (12c), *and acetylspiramycin I* (12d).

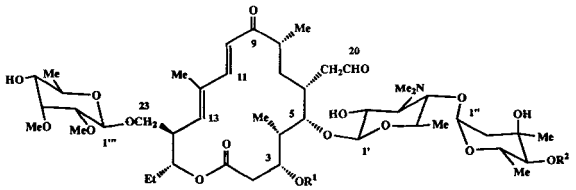
3,4″-di-*O*-acetylspiramycin I (4″-*O*-acetylspiramycin II or simply acetylspiramycin, 12d) exhibited greater *in vivo* activity than spiramycin and received clinical evaluation in Japan [35, 59, 60]. 4″-*O*-Isovalerylspiramycin II was prepared by bioconversion [61], and subsequent work has extended the substituents on the 4″-hydroxyl group to sulphonyl and alkyl groups [62, 63]. Acylation of the 3″-hydroxyl group of spiramycin analogous to the approaches described above yielded derivatives even more effective *in vivo* [64, 65]. However, none of these derivatives apparently possessed enough improvements over available antibiotics to warrant clinical studies.

Selective *N*-demethylation of each amino sugar in spiramycin allowed synthesis of a variety of *N*-acyl derivatives [66]. Hydrolysis of the more acid-labile neutral sugar (mycarose) yields the corresponding neospiramycin factors [67], while further hydrolysis of forosamine yields the forocidin factors [68]. Several 4′-deoxy and 4′-*O*-tetrahydropyranyl derivatives of neospiramycin I have been synthesized, but although some of these had activity comparable to spiramycin, none demonstrated enough significantly superior properties to justify clinical studies [69, 70].

MACROLIDES IN THE TYLOSIN-RELATED FAMILY

TYLOSIN AND RELATED FACTORS

Tylosin (13) (*Figure 5.6*) is the prototype of the second large division of 16-membered macrolides, based upon differences from the leucomycins in aglycone structure. Tylosin is an important veterinary antibiotic produced



13 ($R^1 = R^2 = H$)
14 (R^1 = Ac, R^2 = iVal)
15 ($R^1 = H$, R^2 = 4-$MeOC_6H_4CH_2CO$)

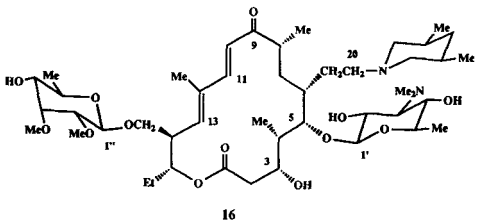
Figure 5.6. Structures of tylosin (13), *AIV-tylosin* (14), *and 4″-O-(4-methoxyphenylacetyl)tylosin* (15).

by *Streptomyces fradiae* [71]. A preliminary conformational study of tylosin has been recently published [72]. Minor factors initially found in culture broths were 3‴-*O*-demethyltylosin (macrocin), demycarosyltylosin (desmycosin), and 20-dihydrotylosin (relomycin) [73]. Other products differing in their degree of glycosylation and/or oxidation state of substituents were later isolated as biosynthetic intermediates or shunt metabolites [74, 75]. This abundance of new compounds has been extensively utilized as starting materials for many diverse semi-synthetic derivatives [6, 26].

ESTERS OF TYLOSIN

The improved *in vitro* spectrum, *in vivo* efficacy, and pharmacokinetics of certain esters of leucomycin-related macrolides logically led to the synthesis of analogous esters of tylosin. 3- and 4″-*O*-acyl derivatives were initially prepared by bioconversions with *Streptomyces thermotolerans*, a producer of carbomycin [76]. From this small group, 3-*O*-acetyl-4″-*O*-isovaleryltylosin (AIV-tylosin) (14) (*Figure 5.6*) was chosen for further study because of its activity against some tylosin-resistant organisms and its improved oral therapeutic and pharmacokinetic effects [61, 77]. AIV-tylosin has now been commercially developed as a new veterinary antibiotic.

Chemical acylation of tylosin subsequently yielded a larger series of esters from which 4″-*O*-(4-methoxyphenylacetyl)tylosin (15) (*Figure 5.6*) has emerged to the stage of preclinical studies due to its activity against certain resistant organisms and its improved metabolic stability [78–80]. Several tylosin-related factors have also been acylated by chemical and biochemical methods [81–84]. In addition to esters, 4″-*O*-alkyl and 4″-deoxy derivatives of tylosin have been prepared, but none was superior to compound (15) [85].



16

Figure 5.7. Structure of tilmicosin (16).

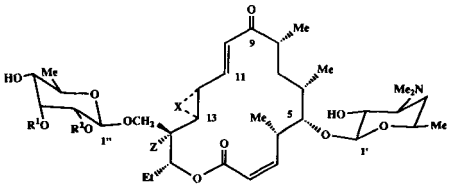
TILMICOSIN

20-Dihydrotylosin (relomycin) has long been known to have less antibacterial activity than tylosin [86]. In contrast, numerous modifications of the aldehyde group in both tylosin and desmycosin were discovered to increase oral efficacy and bioavailability in experimental animals [87]. The optimum antimicrobial spectrum within this series, which especially included *Pasteurella* species responsible for pneumonia in cattle and pigs, was realized from certain 20-dialkylamino-20-deoxo derivatives synthesized by reductive aminations of desmycosin [88, 89]. The 20-deoxo-20-(3,5-dimethylpiperidinyl) derivative of desmycosin, named tilmicosin (16) (*Figure 5.7*), was selected from this series for commercial development. It has recently been launched as a new veterinary antibiotic [90]. Synthesis of a metabolite has been accomplished by a selective *N*-demethylation using $K_3Fe(CN)_6$ [91].

Other reductive aminations of tylosin-related macrolides have been reported, including the synthesis of dimeric moieties which retain antibacterial activity [92, 93]. Other modifications of the aldehyde group include a variety of hydrazone derivatives, reduction products, and *C*-alkylated analogues [83, 94].

MYCINAMICIN GROUP

The mycinamicin complex (also discovered as the AR-5 factors) is produced by *Micromonospora griseorubida* [95]. Structural features that distinguish members of this group include a different amino sugar (desosamine rather than mycaminose), a 2,3-unsaturated lactone, a methyl group rather than a two-carbon substituent at C-6 of the aglycone, and a 14-α-hydroxyl group in some factors. One major component, mycinamicin II (17b) (*Figure 5.8*), has undergone extensive toxicological evaluations [96]. Many individual factors and biosynthetic intermediates have been isolated [97]. Although



17a (X = β-epoxy, Z = H, $R^1 = R^2$ = Me)
17b (X = β-epoxy, Z = OH, $R^1 = R^2$ = Me)
17c (X = single bond, Z = H, $R^1 = R^2$ = Me)

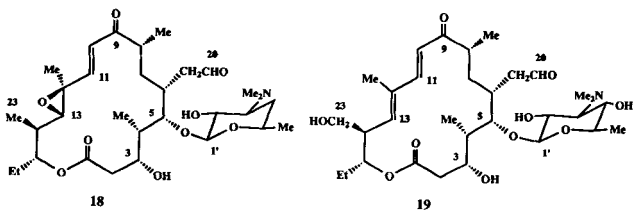
Figure 5.8. Examples of structural variations within the mycinamicin group: mycinamicin I (17a), *mycinamicin II* (17b), *and mycinamicin III* (17c).

results from extensive chemical modifications have not been published, related products have been isolated as co-metabolites or obtained from biosynthetic precursing experiments and bioconversions [97–101].

ROSARAMICIN-TYLONOLIDE GROUP

Rosaramicin (18) (*Figure 5.9*) is produced by *Micromonospora rosaria*; originally named rosamicin, it contains desosamine as its only sugar substituent [102]. Several aldehyde-modified analogues have been isolated from large scale fermentations [103, 104]. A hydrazone of the aldehyde was synthesized which was suitable for X-ray crystallography [105], and tosylhydrazones were reduced to methyl groups by $Cu(BH_4)(Ph_3P)_2$ without affecting other potentially sensitive functionality [106]. Intramolecular cyclizations of the aldehyde have yielded some unusual bicyclic derivatives of the aglycone [107, 108].

Removal of both neutral sugars from tylosin yields 5-*O*-mycaminosyltylonolide (OMT) (19) (*Figure 5.9*); however, the conditions necessary to hydrolyze the relatively stable sugar mycinose require careful control



18 19

Figure 5.9. Structures of rosaramicin (18) *and 5-O-mycaminiosyltylonolide* (19).

because they are sufficiently drastic to simultaneously cause partial decomposition of the aglycone [109]. Consequently, OMT is more conveniently obtained by hydrolysis of the acid-labile sugar mycarose from demycinosyltylosin (DMT), which is isolated from culture broths of mutant strains of *S. fradiae* blocked in the addition of 6-deoxyallose [74].

OMT has been the starting material for many semi-synthetic derivatives [6, 26]. Deoxygenation of mycaminose to desosamine (4′-deoxymycaminose) increased *in vitro* activity approximately two-fold [110], while conversion to its 4′-deoxyfluoro analogue increased activity to a lesser extent [111]. *In vitro* activity was substantially increased by a wide variety of acylation or S_N2 substitution reactions at the 23-hydroxyl group [112–114]. As one example, 23-*O*-benzyl-OMT (TMC-101) was very active but too toxic for development [115]. 23-Amino-23-deoxy-OMT coupled to keyhole limpet haemocyanin was used to raise polyclonal rabbit antibodies which were then developed into an ELISA-based screen capable of detecting macrolides in fermentation broths [116]. Another important discovery was that replacement of the 23-hydroxyl group by certain dialkylamino groups improved activity even against gram-negative bacteria [117–119]. Other modifications reported in this region of the aglycone have included rearrangements, cleavage of substituents, and *C*-alkylidene derivatives [119–122]. In contrast, modifications of the aldehyde group of OMT have generally resulted in reduced activity [89, 114, 123].

A more recently synthesized derivative, 3,4′-dideoxy-OMT (MC-352 or YM-17K) was found to exhibit *in vitro* activity that compared favourably with commercially available macrolides [124–126]. The strong *in vitro* potencies and broad spectrum of macrolides related to OMT and rosaramicin have fueled many efforts to find derivatives with suitable oral efficacy and favourable preclinical features. In addition, new fermentation-derived members of this group such as cirramycin F-1 and F-2, izenamicin, and M-119-a have been isolated [127–129]. However, no OMT-related macrolide has yet emerged as a successful clinical candidate.

OTHER DERIVATIVES

Reported modifications of the dienone include a few 9-dihydro-9-*O*-acyl derivatives which showed limited improvements in activity [130]. Oximes of tylosin such as the analogue of roxithromycin have been made [131]; from this series, the *O*-(*p*-nitrobenzyl) oxime, designated S-5556, exhibited some advantageous properties *in vitro* and *in vivo* [132]. During attempts to prepare the tylosin analogue of azithromycin, the Beckmann rearrangement of the 9-oxime yielded two isomeric ring-enlarged amides [133]. The allylic

9-α configuration was found to be optimal within a small series of 9-deoxo-9-*N*,*N*-dimethylamino derivatives of rosaramicin and OMT [54]. The diene has been reduced to the tetrahydro state and has participated in nucleophilic additions; in the latter situation, thiol reagents proved useful as protecting groups [94, 133–135].

Decarbonylation of the aldehyde was effected either by Wilkinson's catalyst or by decarboxylation after $NaClO_2$ oxidation [87, 136]. Combining the greater *in vitro* activity of 4′-deoxygenation [137] and the favourable pharmacokinetics of aldehyde-modified derivatives [87, 94] led to the synthesis of a new oral macrolide, 19-deformyl-4′-deoxydesmycosin (designated TMC-016) [136, 138]. Other changes at this site have included conversion of the aldehyde to ketones and syntheses of 20-homotylosin and 20-nortylosin [94, 133]. Analogues of a related macrolide, angolamycin, were recently isolated in which the aldehyde group was reduced to either the hydroxymethyl or methyl stage [139].

Oxidation of the hydroxyl group of mycinose yielded an unstable ketone, thereby providing methods for cleaving this sugar [140, 141]; however, the parent demycinosyltylosin is more efficiently obtained from mutant strains of *S. fradiae* [74]. A mild procedure for reductive amination of this unstable ketone with $TiCl_3$ and $NaBH_3CN$ has been developed [142]. Conversion of mycinose in some C-20-modified derivatives into a 3″,4″-unsaturated sugar introduced some *in vitro* antifungal activity into the traditional antibacterial spectrum [143].

A series of 3-*O*-α-L-cladinosyl derivatives was chemically synthesized and some members exhibited favourable characteristics both *in vitro* and *in vivo* [144]. These compounds are hybrids in which the neutral sugar of erythromycin has been incorporated into 16-membered macrolides at a position and configuration analogous to its arrangement in 14-membered macrolides. Some examples previously discussed were structural hybrids either within groups of 16-membered macrolides or between 16- and 14-membered macrolides. Other hybrid structures have been made by more traditional methods of microbial bioconversion [13, 145]. Additional examples of semi-synthetic modifications using the more recent tools of biosynthetic transformation and genetic engineering are given in the next section.

BIOSYNTHETIC AND GENETIC ENGINEERING

Macrolides are secondary metabolites of polyketide origin [146–151]. The apparent similarity between macrolide and fatty acid biosynthesis was

exemplified by using cerulenin, an inhibitor of fatty acid biosynthesis, to prevent formation of native aglycones and to permit incorporation of unnatural aglycones [150]. As one example, precursing a strain of *S. ambofaciens* with an aglycone of tylosin while blocking production of spiramycin with cerulenin yielded hybrid macrolides named chimeramycins, which combined structural elements of both tylosin and spiramycin [152]. Cost and low incorporation limit this methodology to production of relatively small amounts of material, whereas mutant strains blocked in the biosynthesis of their endogenous aglycones overcome many of these problems [153, 154].

Recent studies have demonstrated that macrolide aglycones are assembled on multifunctional enzymes called polyketide synthases which are highly organized in a modular arrangement [155–157]. This conceptual insight permits a more rational approach to biosynthetic modifications by using the tools of molecular biology to introduce precise changes at the genetic level in macrolide-producing organisms [158, 159]. Regulatory genes governing expression of polyketide synthase genes have recently been discovered [160]. The modular proposal reinforces the concept that chain elongation leading to aglycone occurs by successive cycles in which an acyl unit is added to the growing chain followed by elaboration of the intermediate to its final oxidation level prior to addition of the subsequent acyl unit. This mechanism of aglycone assembly had been supported by precursing experiments and isolation of partially assembled putative intermediates [99–101, 161–163].

Genetic manipulations have also altered carbohydrate and acyl substituents. 4″-*O*-isovalerylspiramycin was obtained by cloning the 4″-*O*-acylase gene from the carbomycin-producer *S. thermotolerans* and expressing it in *S. ambofaciens* [164]. The 3-*O*-acylase gene was recently obtained from the midecamycin-producer *S. mycarofaciens* [165]. Combining these two genes with those for tylosin biosynthesis within a single organism could provide the semi-synthetic 3-*O*-acetyl-4″-*O*-isovaleryltylosin by direct fermentation [166]. An alternative synthesis of 19-deformyl-4′-deoxydesmycosin that had used bioconversion for glycosidation suggested that this semi-synthetic macrolide might be obtained by fermentation of an appropriately engineered organism [167]. These examples illustrate the future potential of genetic engineering for preparing novel semi-synthetic macrolides in a cost-efficient manner by fermentation of rationally-modified organisms [168, 169].

BIOLOGICAL AND THERAPEUTIC FEATURES

SPECTRUM OF CLINICAL ANTIMICROBIAL ACTIVITY

In vitro comparisons of josamycin, miokamycin, rokitamycin, and spiramycin with erythromycin and its derivatives showed that they all possessed a similar antimicrobial spectrum that included common susceptible gram-positive organisms plus certain gram-negative cocci and anaerobes [170–172]. *In vitro* activity was increased at higher pH values (8.0 vs. 6.5), but was decreased in some cases by the addition of serum [170–173]. Although these 16-membered macrolides are often slightly less potent than 14-membered macrolides against erythromycin-susceptible bacteria, one distinct advantage of the former is their activity against strains that are inducibly resistant to erythromycin [170–176].

Like their 14-membered relatives, 16-membered macrolides effectively penetrate cells and inhibit intracellular pathogens such as *Chlamydia* and *Legionella* [170, 171, 177, 178]. Miokamycin inhibited some strains of *Mycoplasma hominis* that were poorly susceptible to derivatives of erythromycin [176]. Several 16-membered macrolides had potent activity against *Mycoplasma pneumoniae*, *Mycoplasma genitalium*, and *Ureaplasma urealyticum* [176, 179–181] and were only slightly less active than 14-membered macrolides against *Helicobacter pylori*, an organism increasingly linked to serious gastrointestinal disorders [182].

Although spiramycin is used to treat toxoplasmosis, more potent inhibitors of *Toxoplasma gondii* have been reported [183–185]. In contrast to derivatives of erythromycin, activity was not observed from several 16-membered macrolides against *Mycobacterium avium* complex [186]. Similar trends have been noted with several other bacterial and parasitic pathogens in which 16-membered macrolides have not matched the promising activity shown by erythromycin derivatives [177].

Esters of tylosin such as its 4″-*O*-(4-methoxyphenylacetyl) derivative (YM-133 or IMC-XV) (15) (*Figure 5.6*), are more recent in origin, but they have one advantage with better activity against staphylococci resistant to other macrolides [79, 80, 187]. Removal of the neutral sugar mycinose from tylosin slightly enhances conventional gram-positive activity [123, 188], whereas hydrolysis of the other neutral sugar mycarose is well known to increase activity against gram-negative bacteria [123]. Monoglycosylated macrolides such as rosaramicin exhibit the widest antimicrobial spectrum among macrolide antibiotics [189–192]. The most recent candidate to emerge from the extensive attempts to develop a broad spectrum macrolide by modification of 5-*O*-mycaminosyltylonolide is its 3,4′-dideoxy derivative

(MC-352 or YM-17K), which had improved activity against *Haemophilus influenzae* [124–126]. However, despite many intensive efforts, a macrolide within the tylosin related family has not yet found a position in human medicine.

MECHANISM OF ANTIMICROBIAL ACTION

Although erythromycin has been the most studied, all macrolides are considered to operate by the same overall mechanism in which they penetrate the cell, tightly bind to the 50S ribosomal subunit, and disrupt protein synthesis [193, 194]. Different but overlapping binding sites have been described for different groups of macrolides within the domain of the peptidyltransferase function on the ribosome [195–198]. The requisite uptake of conventional macrolides into the cytoplasm occurs without active transport and depends on the lipophilicity and structural variations of the compounds and the nature of the bacterial outer membrane [199–202]. Since binding studies typically employ ribosomes from *E. coli* that are highly sensitive to macrolides in cell-free systems, the low susceptibility of such gram-negative bacteria to macrolides is indicative of their poor penetration into those bacteria. Disrupting the outer membrane of normally resistant gram-negative bacteria thus leads to greater susceptibility of such mutant strains [203–206].

DEVELOPMENT OF RESISTANCE

Microbial resistance is typically achieved by altering the antibiotic's target site, inhibiting cellular penetration by the antibiotic, increasing its efflux from cells, or converting it to a less active substance [207–209]. The former mechanism is the most common for macrolides and operates via *N*-methylation of an adenine residue in 23S rRNA, causing less ribosomal binding by the antibiotic. Since methylation confers cross-resistance to macrolides, lincosamides and streptogramin B (which have overlapping binding sites), it is termed MLS resistance [209]. MLS resistance is divided into two broad categories: inducible and constitutive [194]. In contrast to erythromycin, 16-membered macrolides do not generally induce resistance to themselves and inhibit uninduced strains. However, a strain of *S. aureus* was recently reported which showed inducible resistance to mycinamicin, a 16-membered macrolide [210]. Although macrolides are generally considered inactive against constitutively resistant strains, unexpected activity against a few such strains of coagulase-negative staphylococci and enterococci was recently noted for miokamycin, spiramycin, and josamycin

[174]. Furthermore, esters of tylosin such as AIV (14) inhibited protein synthesis and bound to tylosin-resistant ribosomes [77]. These results illustrate the dynamics of microbial resistance as antibiotics are developed to combat microorganisms which are continually evolving and acquiring new means of blocking the actions of antibiotics.

Ribosomal methylases have been found in *S. fradiae* and *S. thermotolerans* and are presumed to confer resistance to tylosin and carbomycin, the respective macrolides produced by these organisms [211–213]. Such mechanisms of self-protection suggest that bacterial resistance genes may have been acquired from macrolide-producing organisms [214, 215]. Enzymatic inactivation of macrolides by 2′-*O*-glycosylases, 2′-*O*-phosphorylases, and esterases has been demonstrated in pathogenic bacteria and *Streptomyces*, with substantial variations in substrate specificity [216–221]. The importance of resistance mediated by active efflux of macrolides has recently been recognized [222–226]. Poor intracellular penetration of lipophilic macrolides across the outer membrane of many gram-negative bacteria is likely to be responsible for the lack of activity against these organisms [208, 227].

Oral administration of spiramycin was shown to increase resistance to that antibiotic among strains of faecal bacteria [228]. With the use of antibiotics providing selective pressures on bacteria in favor of resistant strains, the abilities of bacteria to mutate and to transfer genetic material predict that resistance will pose increasingly serious therapeutic challenges. Resistance to macrolide antibiotics is no exception, although the semi-synthetic 16-membered macrolides may play an increasingly important role due to their activity against microbial pathogens resistant to other macrolides [187, 229–232].

ABSORPTION AND DISTRIBUTION IN TISSUES AND FLUIDS

Macrolide antibiotics are administered orally, but many of them exhibit low and/or variable degrees of oral absorption, low serum concentrations, and short half-lives. Consequently, selection of semi-synthetic derivatives for development has often been guided by greater oral bioavailability, longer half-life, and higher and more prolonged concentrations in serum and tissues. The clinical pharmacokinetic parameters of several 16-membered macrolides have recently been reviewed [58, 233, 234].

Macrolides are lipophilic and accumulate within certain cells and tissues at concentrations that exceed serum levels many-fold, resulting in large volumes of distribution. However, high tissue/serum ratios alone do not guarantee intracellular bioavailability or antimicrobial activity [235]. High

concentrations of active antibiotic will eradicate intracellular pathogens, treat extravascular infections, and act as a reservoir for extended release of the antibiotic, thereby giving it a longer *in vivo* half-life; this high intracellular accumulation is most likely responsible for the good post-antibiotic effects of macrolides [172, 236–238]. On the negative side, such highly concentrated distributions may provoke toxicity [239, 240].

Since the lung is among the tissues into which macrolides penetrate well, they are used to treat infections in the respiratory tract, including those caused by intracellular pathogens such as *Legionella*, *Chlamydia*, and *Mycoplasma* [241]. Bronchoalveolar lavage was used as a convenient method to measure high intrapulmonary concentrations of josamycin [242]. Rokitamycin achieved sufficient concentrations of antibiotic in bronchial secretions to treat lower respiratory tract infections [243].

Macrolides are also concentrated inside the phagocytic polymorphonuclear leukocytes (PMNs) and macrophages [244–247]. The migration of PMNs to sites of infection followed by release of intracellular antibiotic has been demonstrated as an *in vivo* drug delivery mechanism [247–249]. Intraphagocytic compounds may exert either positive or negative effects on cell functions. Although this subject has not been completely investigated, especially for 16-membered macrolides, some of them have shown immunostimulatory activity while fewer have exhibited immunosuppressive properties [250–256]. Even less well studied are the effects of antibiotics at sub-inhibitory concentrations on other aspects of the immune or host defence system and their effects on processes such as the anti-inflammatory response [245–247, 255]. In addition to these possible actions of macrolides on the host as biological response modifiers, limited studies have proposed subinhibitory effects on adhesion or virulence of bacteria [257, 258]. However, much more work is required on all of these subjects before any conclusions can be established.

METABOLISM AND PHARMACOLOGICAL INTERACTIONS

Macrolides are metabolized primarily in the liver with their metabolites excreted into bile; metabolism occurs to a lesser degree in the kidneys and lungs [259, 260]. Since macrolides vary widely in their serum and tissue concentrations, half-lives, and active metabolites, knowledge of their metabolism is important for optimizing dosage schedules. Some macrolides also influence the metabolism of certain other drugs, and modified metabolic conditions such as liver disease may alter antibiotic concentrations [260–264]. Because such events can lead to toxicity from either excess antibiotic or adverse drug interactions, metabolism is examined in patients

taking other medications or having serious underlying conditions such as modified liver function. Some macrolides form complexes with and thereby inactivate liver enzymes such as cytochrome P-450 oxidases; however, 16-membered macrolides do so to a lesser degree or not at all [265–267]. Consequently, these macrolides have caused fewer drug interactions with compounds such as theophylline, a frequently used bronchodilator metabolized by cytochrome P-450 enzymes [260, 268].

Several 16-membered macrolides form metabolites which retain antimicrobial activity. As discussed above, 3″-esters such as rokitamycin and miokamycin produce prolonged concentrations of antibiotic *in vivo* due to the facile 3″- to 4″-*O*-acyl migration that follows enzymatic removal of the original 4′-ester [34, 269, 270]. Following a different approach to overcome the lability of 4″-esters, specific 4″-*O*-acyl derivatives of tylosin were selected from the series of esters (15) based upon their greater stability toward liver enzymes [80]. Although esterases play the most prominent role in metabolism of 16-membered macrolides, other mechanisms such as oxidative hydroxylation, *N*-demethylation, reduction, and hydrolysis of sugars have been reported for various compounds [91, 96, 115, 259, 270–272].

CLINICAL EXPERIENCE

16-Membered macrolides are generally administered orally to treat infections caused by susceptible pathogens in the respiratory and genital tracts and skin and soft tissues, indications similar to those for erythromycin [35, 273]. Since macrolides exhibit good penetration of tissues and high intracellular concentrations, they are effective against intracellular organisms residing within these sites, such as *Legionella*, *Chlamydia*, *Mycoplasma*, and *Ureaplasma* [274]. Although 16-membered macrolides have not yet been developed in the U.S., josamycin is used in many other countries, miokamycin and rokitamycin are becoming more widely available, and others have established themselves in certain niches. Spiramycin shows greater *in vivo* efficacy than expected from its *in vitro* potency; in addition to the usual indications, it is used in Europe to treat toxoplasmosis [58, 275]. It has also been used to treat dental infections since it inhibits many bacteria implicated in oral diseases such as caries and chronic periodontitis [276, 277]. Rosaramicin had been under clinical investigation which included treatment of infections in the genitourinary tract, but its development has since ended [278–280]. Overall, however, 16-membered macrolides have not generally received as much attention as erythromycin and its semi-synthetic derivatives, apparently because no individual member of the former group

is perceived to possess as many of the advantageous features, both *in vitro* and *in vivo*, that are ascribed to the latter group.

All macrolides are regarded as relatively safe antibiotics whose principal side-effects are gastrointestinal disturbances ranging from mild upset to severe pain [281]. Gastrointestinal motility in conscious dogs is not affected by 16-membered macrolides, in contrast to the substantial effects provoked by 14-membered macrolides [282–284]. These different effects on motility have recently been confirmed in clinical studies of miokamycin compared with several 14-membered macrolides [285, 286]. Less frequent side-effects such as hypersensitivity and cutaneous reactions have been mentioned [35, 287, 288].

VETERINARY FEATURES AND EXPERIENCE

Tylosin and spiramycin are the natural 16-membered macrolide antibiotics most widely used in veterinary medicine as therapeutic agents and feed additives [4, 289]. Their utility is due to the same features described above for clinical applications, such as a potent antimicrobial spectrum covering gram- positive bacteria, selected gram-negative organisms, and intracellular species such as *Mycoplasma* and *Ureaplasma* [289–291]. They are orally bioavailable, well distributed in many body fluids except the central nervous system, and achieve high and prolonged concentrations in tissues such as lung, liver, kidney, spleen, and the genital tract. They are safe and effective agents against tissue infections, abscesses, and diseases in the respiratory, prostatic and integumentary systems of food and companion animals [292].

One of the newer semi-synthetic macrolides for veterinary use is 3-*O*-acetyl-4″-*O*-isovaleryltylosin (AIV-tylosin) (15) (*Figure 5.6*). Its *in vitro* spectrum is similar to tylosin, but includes activity against some tylosin-resistant organisms; *in vivo*, it attains higher serum concentrations than tylosin [61, 77]. It has been reported to prophylactically control *Mycoplasma gallisepticum*-induced air sac lesions and *Treponema hyodysenteriae*-swine dysentery after oral administration [293, 294].

A second new semi-synthetic macrolide is tilmicosin, which inhibits a variety of animal pathogens including species of *Pasteurella* [295]. It is effective for both treatment and prophylaxis of respiratory infections in cattle after a single injection due to its long *in vivo* half-life and has been registered for the treatment of bovine respiratory disease [296–299]. Tilmicosin is also orally bioavailable and has treated pneumonia due to *Pasteurella* and *Actinobacillus* in pigs when incorporated into feed [300, 301].

SUMMARY

The fermentation-derived 16-membered and 14-membered macrolides have been equally productive sources of semi-synthetic derivatives which have significantly extended the utility of the macrolide class as important antibiotics. New derivatives, prepared by both chemical and biochemical methods, have exhibited a variety of improved features, such as an expanded antimicrobial spectrum, increased potency, greater efficacy, better oral bioavailability, extended chemical and metabolic stability, higher and more prolonged concentrations in tissues and fluids, lower and less frequent dosing, and/or diminished side-effects [302]. However, even more improvements are both achievable and necessary if problems such as resistance to existing antibiotics continue to rise [303, 304]. Newer semi-synthetic macrolides which satisfy these important needs should be anticipated as the contributions from new fields such as genetic engineering of macrolide-producing organisms and more powerful computational chemistry are combined with the more traditional disciplines of chemical synthesis, bioconversions, and screening fermentation broths.

ACKNOWLEDGEMENTS

The author sincerely appreciates the numerous literature searches conducted by Ms. L. W. Crandall and the invaluable computer support provided by Dr. G. G. Marconi.

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Progress in Medicinal Chemistry – Vol. 31, edited by G.P. Ellis and D.K. Luscombe

6 β-Lactamases: Targets for Drug Design

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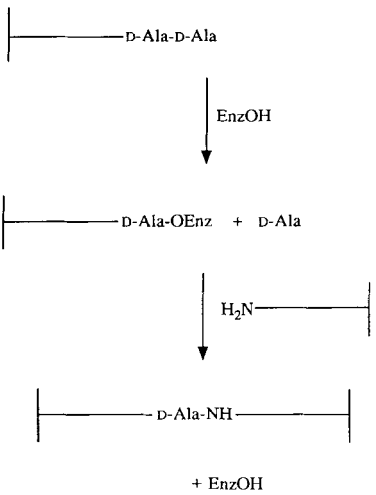
INTRODUCTION

It is now over 60 years since the serendipitous discovery of penicillin by Fleming [1], and approximately 50 years since its chemotherapeutic potential was first recognised by Florey and co-workers [2] at Oxford. Since the end of World War II, the great commercial and academic interest in β-lactam antibiotics has led to a wide array of antibacterial agents of immense chemotherapeutic value.

β-Lactam antibiotics exert their antibacterial activity by interacting with bacterial enzymes, and in doing so, inhibiting bacterial cell wall synthesis. The growth and division of bacterial cells necessitates production of new cell wall material in which the final step is the cross-linking of specific peptide chains to form a peptidoglycan matrix (*Scheme 6.1*). Cross-linking is mediated by several enzymes, referred to as penicillin-binding proteins (PBP's) or transpeptidases, and all these enzymes are believed to contain serine at the active site. Acylation of the serine hydroxyl group is believed to be the key step in the cross-linking process [3]. β-Lactam antibiotics interfere with this step by simulating the D-Ala-D-Ala portion of the peptide substrate, and themselves acylate the serine hydroxyl group, with the opening of the β-lactam ring (*Scheme 6.2, step B*). The acylated enzyme then rearranges to a stable form and thus permanently deactivates the enzyme (*Scheme 6.2, step D*). The net effect is that the transpeptidase is prevented from exercising its normal function of completing the peptide cross-linkages

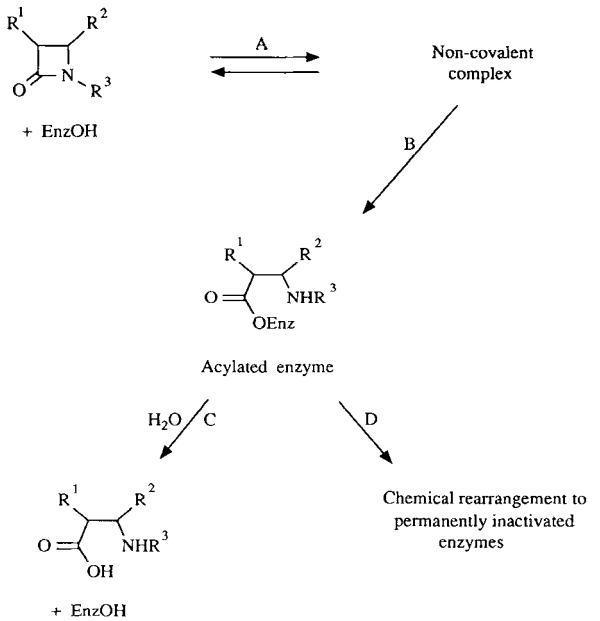


Scheme 6.1. Peptidoglycan cross-linking by transpeptidase enzyme.

in the cell wall. The cell wall weakens as the cell grows and eventually bursts, resulting in cell death.

The widespread use of penicillins and cephalosporins has, however, resulted in the emergence of bacterial resistance attributed to β-lactamases, a family of bacterial enzymes that catalyse the hydrolysis of the β-lactam ring.

With the exception of one rather uncommon class of zinc-requiring β-lactamase [4], initial non-covalent binding at the active site frequently leads to acylation of a serine hydroxyl group by the β-lactam (*Scheme 6.2, step B*) which, irrespective of the ultimate fate of the protein, results in irreversible inactivation of the β-lactam. A β-lactam is a substrate for the β-lactamase if acylation is followed by rapid hydrolysis of the serine ester linkage (*Scheme 6.2, step C*), which regenerates the active enzyme and at the same time a ring-opened form of the β-lactam, which is antibacterially inactive; hence the term β-lactamase. Alternatively, formation of the acylated enzyme may be followed by chemical rearrangement to a permanently inactivated β-lactamase (*Scheme 6.2, step D*); in this situation the β-lactam agent acts as an inhibitor of the β-lactamase [5, 6].



Scheme 6.2. Interaction of β-lactams with serine proteases. [6]

In combination with β-lactamase-susceptible antibiotics, β-lactamase inhibitors protect the antibiotic from inactivation by the β-lactamase enzymes, thereby producing a synergistic effect against β-lactamase-producing bacteria and extending the spectrum of activity of the antibiotic.

The present review discusses the identification of such β-lactamase inhibitors from natural sources as well as some of the more successful efforts towards developing potent, broad-spectrum inhibitors of bacterial β-lactamases, based upon the β-lactam structure, for clinical utility. It is not intended to be a comprehensive review, nor is it intended to encompass non-β-lactam inhibitors.

CLASSIFICATION OF β-LACTAMASES

The existence of β-lactamases was first noted as early as 1940, when Abraham and Chain reported that crushed cells of *Escherichia coli* were capable of destroying penicillin [7]. They concluded that the destruction resulted from the action of an enzyme that they named penicillinase. By 1944 Kirby had demonstrated that penicillin-resistant strains of *Staphylococcus aureus* caused inactivation of penicillin, and it was evident that the resistance of *Staphylococci* to penicillin was from β-lactamase activity [8]. Many different β-lactamases are now known, with differing specificities for the various types of β-lactam [9]. They occur in both Gram-positive and Gram-negative bacteria, as well as in blue-green algae and yeasts. Some β-lactamases are plasmid-mediated, in which case the genetic information necessary for their production may be readily transferred between bacterial species. Other β-lactamases are chromosomally mediated, and a low level of these is believed to occur very widely among Gram-negative bacteria. In some species, exposure to certain β-lactams may induce formation of much larger amounts of 'class I' cephalosporinase-type chromosomally mediated β-lactamases. Fears have been expressed that widespread use of the newer broad-spectrum β-lactams may lead to the proliferation of highly resistant strains of *Enterobacter*, *Serratia* and *Pseudomonas* capable of producing very large amounts of cephalosporinase [10].

β-Lactamases have been classified in a number of different ways. β-Lactamases from Gram-positive organisms such as staphylococcal organisms are referred to in general as penicillinases, or penases [11]. Gram-negative bacteria, however, produce a much greater diversity of β-lactamases and therefore require a more complex classification scheme.

Until recently, the most frequently used scheme for the classification of Gram-negative β-lactamases was that of Richmond and Sykes [11, 12]. In

this scheme, five broad enzyme classes were initially identified on the basis of substrate profile and inhibition studies, and each group contained a number of enzyme types.

Class I enzymes, which have been further subdivided Ia to Id, are predominantly active against cephalosporins.They are characteristically produced by strains of *Escherichia coli, Enterobacter* species, *Morganella, Proteus vulgaris, Pseudomonas, Citrobacter, Klebsiella* and *Serratia* species. The genetic information of these enzymes is chromosomally mediated and enzyme production may be constitutive or inducible.

Class II enzymes are principally active against penicillins and are chromosomally-mediated. They are found in *Proteus mirabilis* and *E. coli.*

Class III enzymes are the plasmid-mediated TEM*-type β-lactamases which have approximately equal activity against penicillins and cephalosporins; they are sensitive to inhibition by cloxacillin and resistant to inhibition by *p*-chloromercuribenzoate. They are commonly found in strains of *E. coli* and *Pseudomonas*, as well as *Haemophilus, Neisseria, Salmonella* and *Shigella.* The SHV-1 and HMS enzymes also belong to this class of enzyme and together they are the most commonly encountered β-lactamases worldwide.

Class IV enzymes have a similar substrate profile to the enzymes of class III; they are however resistant to inhibition by cloxacillin and sensitive to inhibition by *p*-chloromercuribenzoate. The most important class IV β-lactamases are those produced by strains of *Klebsiella*, and are invariably constitutive and of chromosomal origin.

Class V enzymes have a 'penicillinase' profile, including activity against cloxacillin; they are resistant to inhibition by *p*-chloromercuribenzoate.This class of enzymes includes the oxacillin-hydrolysing enzymes, OXA-1, OXA-2, and OXA-3, and the *Pseudomonas*-specific carbenicillin hydrolysing enzymes, PSE-1, PSE-2, PSE-3, and PSE-4. They are plasmid-mediated enzymes and are found in *E. coli, Pseudomonas* and *Serratia* species.

A sixth group (class VI) has recently been added for the β-lactamases produced by *Bacteroides* species [13]. They hydrolyse cephalosporins better than penicillins and are inhibited by either cloxacillin or carbenicillin.

An alternative classification of β-lactamases is that based on amino-acid sequence homology and molecular size. Three classes of enzymes are recognised, namely A, B and C [14, 16].

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*TEM enzymes were named after a young girl named Temoniera, from whom they were first isolated [13]; SHV enzymes are sulphydryl variable; HMS enzymes were named after Hedges, Matthew and Smith; OXA enzymes are so named as they are oxacillin-hydrolyzing; PSE enzymes are Pseudomonas specific enzymes.

Class A comprises a set of enzymes of approximately 30,000 Da that are preferentially active against penicillins and share considerable homology with one another. The key amino-acid residue in members of this class is serine-70. Within this class are the penicillinases of the Gram-positive organisms, *Bacillus licheniformis* and *S. aureus* and the TEM-type broad-spectrum β-lactamases from Gram-negative bacteria.

The class C β-lactamases include the chromosomal cephalosporinases of Gram-negative bacteria. They are large molecules of approximately 39,000 Da and show no sequence homology with the other classes. The amino-acid residue directly implicated in enzyme action of this class has been identified as serine-80.

In contrast to class A and class C enzymes, class B enzymes are metallo-thio enzymes and this class is restricted to *Bacillus cereus* type II β-lactamase. They have a molecular mass of approximately 23,000 Da.

The most recent classification of β-lactamases is that of Bush [17], who proposed a modification of the Richmond-Sykes system based primarily on biochemical characteristics, using substrate and inhibitor profiles in addition to physical data. This system, which also includes β-lactamases from Gram-positive bacteria, seems likely to become the preferred system for the classification of β-lactamases in the future.

The Bush classification system is divided into four main groups. Group 1 β-lactamases are those enzymes that preferentially hydrolyse cephalosporins and are not inhibited by 10 μM clavulanic acid; they correlate primarily with the Richmond and Sykes class Ia, Ib and Id β-lactamases. Group 2 β-lactamases include a variety of enzymes that are all inhibited by clavulanic acid. Group 2a enzymes are the classical penicillinases, including those enzymes from Gram-positive bacteria. Group 2b enzymes are the traditional broad-spectrum β-lactamases, such as the TEM-1, TEM-2 and SHV-1 enzymes. Group 2b′ includes many β-lactamases related to those of group 2b, but with the ability to hydrolyse the 'extended broad-spectrum' β-lactam antibiotics, such as cefotaxime, ceftazidime, or aztreonam. Groups 2c and 2d include those penicillinases that hydrolyse carbenicillin or cloxacillin, respectively. Group 2c encompasses the PSE-1, PSE-3 and PSE-4 enzymes, while group 2d includes the OXA-1 and PSE-2 enzymes. Group 2e includes a unique group of cephalosporinases that are inhibited by clavulanic acid and resemble penicillinases in immunological properties. In group 3 are the metallo-enzymes, such as *Bacillus cereus* II, and group 4 includes a variety of penicillinases that are not inhibited by clavulanic acid.

The clinically most important β-lactamases are regarded as the Gram-positive penicillinases, the Richmond-Sykes class I cephalosporinases and the class III plasmid-mediated TEM-1 enzymes. Wherever possible, the

activity of the β-lactamase inhibitors against these enzymes will be indicated in the present review.

DETERMINATION OF β-LACTAMASE INHIBITORY ACTIVITY

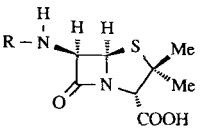
The β-lactamase inhibitory activity of a particular compound may be determined against isolated enzymes in a cell-free assay as well as against whole bacterial cells in combination with an appropriate β-lactamase-sensitive β-lactam antibiotic.

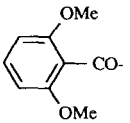
In the former assay, the β-lactamase inhibitory activity is expressed as the I_{50} value, which is the concentration of inhibitor giving 50% inhibition of substrate hydrolysis by the test enzyme under defined conditions. The values are usually determined with and without pre-incubation of the inhibitor with the enzyme. With pre-incubation, the inhibitor is allowed to react with the enzyme for a period of 5 or 15 minutes under physiological conditions before adding the substrate to measure residual enzyme activity. Without pre-incubation, the enzyme is usually added to pre-mixed inhibitor and substrate. For inhibitors which progressively inhibit the β-lactamase and form an acyl-enzyme with some degree of stability, the I_{50} values are lower with pre-incubation than those without pre-incubation. Compounds giving similar I_{50} values are usually acting in a competitive manner. I_{50} values are only constant under defined conditions and are influenced by the length of pre-incubation, the type of substrate and the concentrations of the substrate. In some cases I_{50} values have been determined by using penicillins and cephalosporins such as penicillin G and cephaloridine as substrate. The most convenient method of I_{50} determination however utilizes the chromogenic substrate, nitrocefin [18].

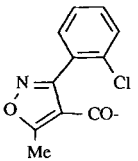
Synergistic activity is determined by measuring the minimum inhibitory concentration (MIC) of a β-lactamase-sensitive β-lactam antibiotic against a β-lactamase-producing strain of bacteria, both in the presence of the test inhibitor and alone. Synergy is generally believed to have occurred when the MIC value of the antibiotic in the presence of the inhibitor is reduced by at least four-fold compared with that of the antibiotic alone [19]. The minimum inhibitory concentration is the lowest concentration of antibiotic required to prevent visible growth of bacteria after incubation for 18 hours at 37°C and is usually determined in microtitre plates by serial dilution of the antibiotic in broth, followed by addition of a fixed concentration of the inhibitor and the organism (approx. 2×10^6 colony-forming units per ml).

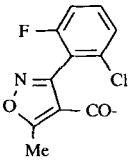
The synergy test is the more stringent of the two tests as synergistic activity is dependent upon the test compound being able to penetrate the

outer membrane of Gram-negative bacteria in order to inhibit the periplasmic β-lactamases.

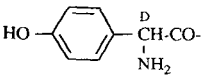


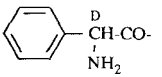
(1) R =  Methicillin

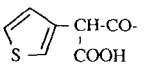
(2) R =  Cloxacillin

(3) R =  Flucloxacillin

(4) R = $PhCH_2CO$- *Penicillin G* (Benzylpenicillin)

(5) R =  Amoxycillin

(6) R =  Ampicillin

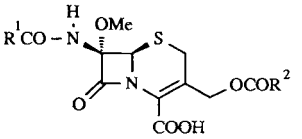
(7) R =  Ticarcillin

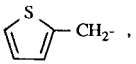
(8) R = H 6-Aminopenicillanic Acid (6-APA)

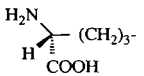
IDENTIFICATION OF β-LACTAMASE INHIBITORS FROM NATURAL SOURCES

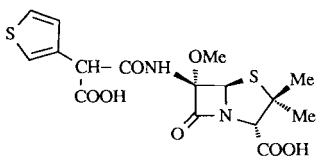
The increasing number of β-lactamase producing bacteria capable of inactivating β-lactam antibiotics became a major concern in β-lactam therapy and prompted considerable effort in order to overcome this problem.

One approach has been to modify the structure of the β-lactam antibiotic so as to increase its stability and thus confer resistance to β-lactamases, whilst retaining its inhibitory activity against transpeptidases. This was achieved by introducing sterically crowded 6-acyl substituents, as in methicillin (1) and the isoxazolyl penicillins (2) and (3) [20], or by the introduction of a methoxy substituent to the α-face of the β-lactam, as in cefoxitin (9) [21] and temocillin (11) [22]. The two latter compounds were a direct result of the discovery of the β-lactamase stable cephamycins (10), isolated by fermentation of *Streptomyces* in 1970 by Merck and Lilly [23].



(9) $R^1 =$  $R^2 = NH_2$ Cefoxitin

(10) $R^1 =$  $R^2 = NH_2$,etc. Cephamycins



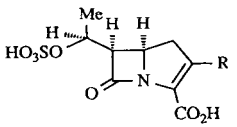
(11) Temocillin

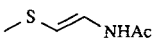
The second approach to combat the action of β-lactamases was to identify an agent that was capable of inhibiting the bacterial enzyme, and in doing so to protect the β-lactam antibiotic from destruction.

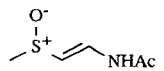
Encouraged by the discovery that methicillin (1) and cloxacillin (2) inhibited a small number of β-lactamases [24, 25], Beecham scientists initiated a screening programme for the detection of naturally occurring β-lactamase inhibitors [26]. The assay involved seeding an agar plate containing Penicillin G (4) with a β-lactamase producing strain of *Klebsiella aerogenes*. The test solution was then introduced into wells in the agar and the plate was incubated overnight at 37°C. Solutions containing a diffusible β-lactamase inhibitor gave zones of inhibition around the wells, resulting from the protection of the penicillin present in the agar. In the absence of β-lactamase inhibitor, bacterial growth occurred as a result of the inactivation of the pencillin by the β-lactamase.

CARBAPENEMS

Application of the above screen led to the detection of a number of β-lactamase inhibitors from a number of strains of *Streptomyces olivaceus*. They were identified as the carbapenem derivatives, MM 13902 (12), MM 4550 (13) and MM 17880 (14), known collectively as the olivanic acids [27, 28]. Not only are they potent β-lactamase inhibitors, but are also powerful antibacterial agents. Subsequently, a whole series of carbapenem derivatives



(12) R =  MM 13902

(13) R =  MM 4550

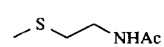
(14) R =  MM 17880

Table 6.1. β-LACTAMASE INHIBITORY ACTIVITY OF THE NATURALLY OCCURRING OLIVANIC ACID DERIVATIVES [29]

Compound	I_{50} *(μg/ml)*			
	S.a. Russell[a]	*E.co. JT4*[a] *(III)*[c]	*K.a. E70*[a] *(IV)*	*E.cl. P99*[b] *(I)*
MM 4550	0.015	0.001	0.002	0.004
MM 13902	0.050	0.020	0.050	0.001
MM 17880	0.075	0.010	0.060	0.001

[a]Substrate, benzylpenicillin with 5 min pre-incubation; [b]substrate, cephaloridine with 5 min pre-incubation; [c]enzyme classification based upon Richmond-Sykes.

Abbreviations for tables: *C.d., Citrobacter diversus; C.f., Citrobacter freundii; E.ae., Enterobacter aerogenes; E.cl., Enterobacter cloacae; E.co., Escherichia coli; K.a., Klebsiella aerogenes; K.o., Klebsiella oxytoca; K.p., Klebsiella pneumoniae; M.m., Morganella morganii; P.m., Proteus mirabilis; P.v., Proteus vulgaris; Ps.a., Pseudomonas aeruginosa; Se.m., Serratia marcescens; S.a., Staphylococcus aureus.*

have been isolated from natural sources and many are potent inhibitors of β-lactamases. Their occurrence and biological properties are the subject of several recent reviews [9, 18, 29, 30].

The inhibitory activity of the olivanic acids is shown in *Table 6.1.* Synergism with penicillins and cephalosporins against β-lactamase producing bacteria can be shown with these compounds (*Table 6.2*) although the effects are not as pronounced as one might anticipate from the β-lactamase inhibitory data. The amount of olivanate derivative, however, that may be

Table 6.2. SYNERGISM BETWEEN AMOXYCILLIN AND OLIVANIC ACID DERIVATIVES AGAINST β-LACTAMASE PRODUCING BACTERIA [19]

Compound combination	*Inhibitor conc. (μg/ml)*	*MIC (μg/ml)*			
		E.co. JT39	*K.a. A*	*P.m. 889*	*S.a. Russell*
Amoxycillin alone	–	>1000	250	>1000	250
+ MM 4550	1.0	250	5.0	100	1.0
+ MM 13902	0.05	>1000	100	>1000	5.0
+ MM17880	0.05	1000	50	>1000	5.0

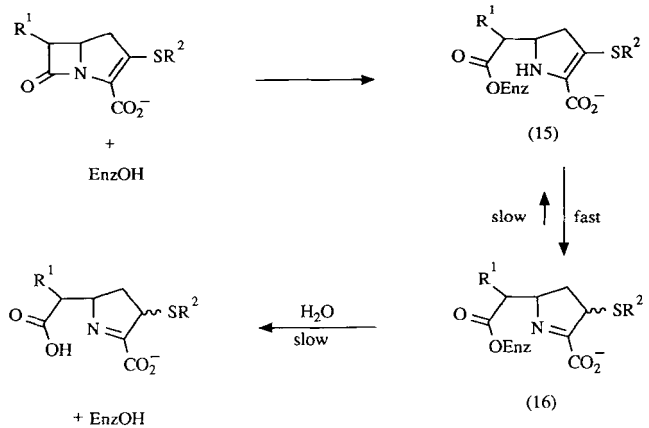
For abbreviations, see footnote to *Table 6.1.*

added as an inhibitor is small because of the intrinsic activity of the olivanate as an antibiotic, which makes the evaluation of the series as potential β-lactamase inhibitors rather complex [19, 30].

Mechanism of action of carbapenems

Many clinically important β-lactamases are serine proteases that catalyse β-lactam hydrolysis by a double displacement mechanism involving a covalent acyl-enzyme intermediate. Inhibitors of these enzymes exert their effect by the formation of a stable acyl-enzyme complex. In most cases, this is as a result of changes that take place in the acyl residue after interaction with the enzyme, that is, the inhibitors are mechanism-based. In other cases, the inhibition of β-lactamases may merely be due to the formation of a relatively stable covalent acyl-enzyme complex without additional alteration [31].

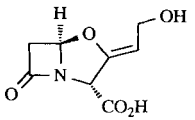
The carbapenems are mechanism-based inhibitors which involve acylation of the active-site residue and subsequent rearrangement to a more stable acyl-enzyme species. Knowles and co-workers [32, 33] have demonstrated that the progressive inhibition of the TEM β-lactamase by the olivanic acids is due to the rearrangement of the Δ^2-pyrroline intermediate (15) to the tautomeric and thermodynamically more stable Δ^1-pyrroline (16) (*Scheme 6.3*). The resultant acyl-enzyme complex is believed to be stable to subsequent hydrolytic breakdown, thereby disrupting the catalytic activity of the enzyme.



Scheme 6.3. Mechanism of action of carbapenems [32, 33, 37].

CLAVULANIC ACID

A short time after the isolation of the olivanic acids from *Streptomyces olivaceus*, Beecham scientists detected another potent β-lactamase inhibitor in a culture of *Streptomyces clavuligerus*. This inhibitor was isolated and characterized as clavulanic acid (17) [34, 35]. Whilst this compound has only



(17) Clavulanic Acid

weak antibacterial activity, it has been shown to be a potent inhibitor of many clinically important β-lactamases [35, 36]. The inhibitory activity of clavulanic acid against representative β-lactamases is shown in *Table 6.3*. It

Table 6.3. β-LACTAMASE INHIBITORY ACTIVITY AND SYNERGISTIC ACTIVITY OF CLAVULANIC ACID IN COMBINATION WITH AMOXYCILLIN [35, 36]

Compound combination	*I_{50} (μg/ml)*				*MIC (μg/ml) geometric mean*			
	S.a.[b] *Russell*	*E.cl*[c] *P99 (I)*[e]	*P.m.*[b] *C889 (II)*	*E.co.*[b] *JT4 (III)*	*S.a. (35)*[a]	*Proteus sp (23)*[a,d] *(I,II)*	*E.co. (100)*[a] *(III)*	*K.a. (45)*[a] *(IV)*
Clavulanic acid	0.06	10	0.03	0.08	17.1	62.9	24.8	33.2
Amoxycillin alone	–	–	–	–	197	433	>5000	315
Amoxycillin + 1.0 μg/ml Clavulanic acid	–	–	–	–	0.6	11.6	94.5	1.75
Amoxycillin + 5.0 μg/ml Clavulanic acid	–	–	–	–	0.12	4.2	13.2	0.89

[a]No. of strains; [b]substrate, penicillin G; [c]substrate, cephaloridine; [d]*Proteus mirabilis* and *Proteus vulgaris* strains; [e]Richmond and Sykes classification of enzymes. For abbreviations, see footnote to *Table 6.1*.

was found to be a potent inhibitor of the β-lactamases derived from Gram-positive bacteria, e.g. *Staphylococcus aureus*, and the β-lactamases, especially the class II, III, IV and V types, as defined by Richmond and Sykes, from Gram-negative organisms. β-Lactamases not readily inhibited by clavulanic acid are the class I type cephalosporinases.

It has also been shown that clavulanic acid and amoxycillin (5) have pronounced synergistic activity against β-lactamase producing strains of *Staphylococcus aureus, Klebsiella aerogenes, Proteus mirabilis* and *Proteus vulgaris, Bacteroides fragilis, Moraxella catarrhalis* and certain strains of *Escherichia coli, Shigella, Haemophilus influenzae, Neisseria gonorrhoeae, Salmonella, Serratia* and *Pseudomonas* (namely those producing plasmid-mediated enzymes, e.g., TEM type β-lactamases) (*Table 6.3*).

Similarly, good synergistic effects are found with ticarcillin (7) plus clavulanic acid [36]. In fact, a combination with ticarcillin has very broad spectrum activity because ticarcillin itself shows good activity against organisms producing β-lactamases poorly inhibited by clavulanic acid.

The in vivo activity of a combination of clavulanic acid and amoxycillin is shown in *Table 6.4* [30]. This β-lactamase inhibitory activity, together with useful pharmacokinetic properties, has resulted in the development of Augmentin (potassium clavulanate in combination with amoxycillin) for oral and parenteral use and Timentin (potassium clavulanate plus ticarcillin) for parenteral use, against a wide range of bacterial infections*.

Table 6.4. *IN VIVO* SYNERGISTIC ACTIVITY OF AMOXYCILLIN IN COMBINATION WITH CLAVULANIC ACID [30]

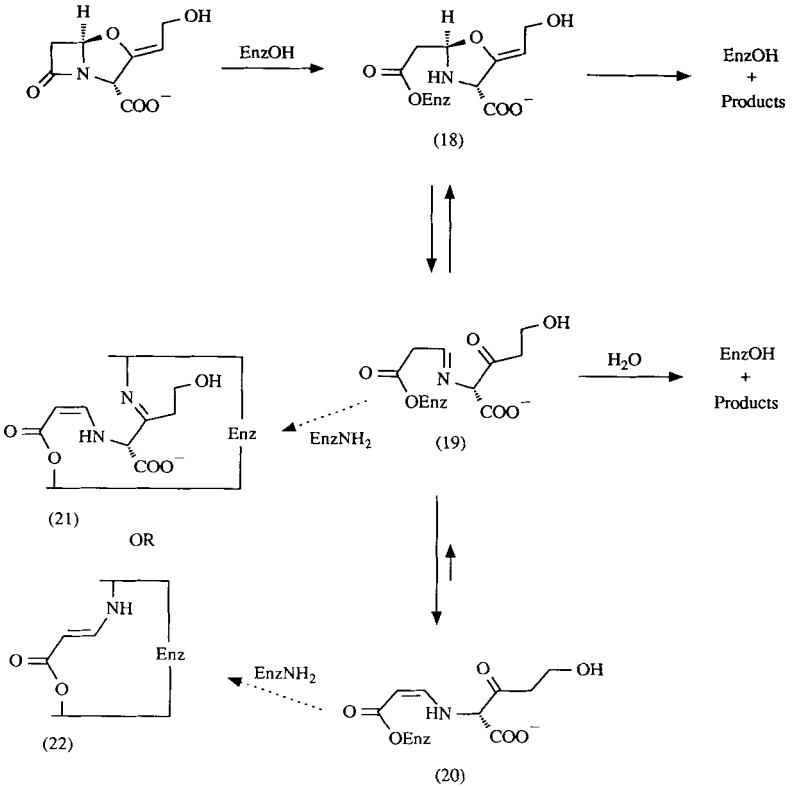
Compound combination	*Amoxycillin CD_{50}[a] (mg/kg × 2)*			
	P.m. C889 (II)[c]	*K.a. I 112*	*K.a. Ba95*[b]	*E.co. JT39*[b] *(III)*
Amoxycillin alone	>1000	>1000	>1000	>1000
Amoxycillin + 5 mg/kg Clavulanic acid	14	6.6	25	7.7

[a]CD_{50}, Curative dose for 50% of mice following subcutaneous dosing 1h and 5h post infection; [b]strains producing plasmid-mediated β-lactamase; [c]Richmond-Sykes classification of enzymes. Clavulanic acid alone was ineffective at 50 mg/kg. For abbreviations, see footnote to *Table 6.1*.

*Augmentin and Timentin are Trademarks of SmithKline Beecham p.l.c.

Mechanism of action of clavulanic acid

Clavulanic acid is also a mechanism-based inhibitor and its mode of action is believed to involve ring opening of the initially formed acyl-enzyme complex (18) to the keto-derivative (19), which may then tautomerise to the hydrolytically more stable β-amino-acrylate (20) (*Scheme 6.4*). This transiently inhibited form may hydrolyse to re-release active enzyme or react further with the enzyme to produce irreversibly inhibited forms. It has been shown that approximately 115 molecules of clavulanic acid are destroyed per molecule of enzyme before the β-lactamase is irreversibly inactivated. Whilst irreversibly inactivated forms are known to exist, the nature of these products is not yet known. Possible structures are (21) and



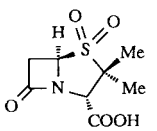
Scheme 6.4. Mechanism of action of clavulanic acid [31,37–9].

(22), arising from the reaction of an enzymic amine, such as lysine, with either (19) or (20) [31, 37–39].

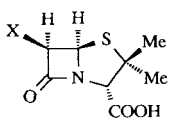
SEMI-SYNTHETIC β-LACTAMASE INHIBITORS

The isolation of the olivanic acids and clavulanic acid from natural sources stimulated a worldwide search for β-lactamase inhibitors with improved biological properties. Whilst much effort was devoted to the chemical modification of the olivanic acids and clavulanic acid, perhaps the greatest success has been achieved by the chemical manipulation of 6-aminopenicillanic acid (6-APA) (8) and penicillin G (4). The purpose of this review is to describe some of the successful efforts that have been made to design and synthesize potent inhibitors of bacterial β-lactamases from these readily available chiral synthons.

The first notable success was that of penicillanic acid sulphone (sulbactam; CP-45,899) (23) [40], which was synthesized by Pfizer chemists from 6-APA (8) and shown to possess potent β-lactamase inhibitory activity. Many other semi-synthetic β-lactamase inhibitors have been

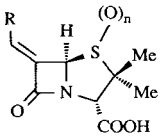


(23) Sulbactam



(24) X = Br

(25) X = I

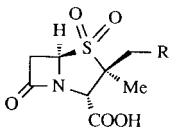


(26) R = MeO, n = 0

(27) R = MeCO, n = 0

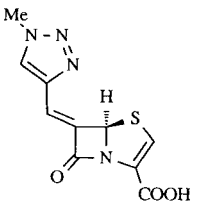
(28) R =  , n = 2

identified and include the 6-β-halopenicillanic acid derivatives (24) and (25) [41–43], 6-methoxymethylene penicillanic acid (26) [44], 6-acetylmethylene penicillanic acid (27) [45], 6-pyrid-2-ylmethylenepenicillanic acid sulphone (28) [46], the 6-β-chloromethyl penam derivative (29) [47], and tazobactam (YTR-830) (30) [48–50]. Most recently, SmithKline Beecham scientists have described the synthesis and biological evaluation of a series of 6-heterocyclylmethylene penem derivatives. A member of this group, the 6-(*Z*)-(1-methyl-1,2,3-triazol-4-ylmethylene)penem (BRL 42715) (31) [51], was reported to be an extremely potent inhibitor of β-lactamases, the spectrum and degree of activity observed representing a significant improvement over other known inhibitors, including clavulanic acid, sulbactam and tazobactam.



(29) R = Cl

(30) R =  , Tazobactam



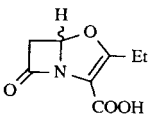
(31) BRL 42715

CLAVULANIC ACID DERIVATIVES

The chemical modification of clavulanic acid has been investigated extensively and is the subject of several reviews [18, 52–54]. To date, this research has failed to provide a clinical successor to clavulanic acid.

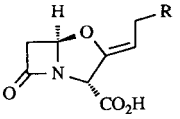
The inhibitory activity of some representative examples is shown in *Table 6.5* [30]. Generally, the potency of these derivatives is of the same order, or less than that of the parent compound.

The oxapenem derivative (32) however displayed a different spectrum of



(32)

Table 6.5. β-LACTAMASE INHIBITORY ACTIVITY (I_{50} μg/ml) OF CLAVULANIC ACID DERIVATIVES AND ANTIBACTERIAL ACTIVITY OF AMPICILLIN IN THE PRESENCE OF THESE DERIVATIVES (MIC μg/ml)[a] [30]



Compound R	*S.a. Russell*		*E.co. (TEM) (III)*[c]		*K.a. E70 (IV)*		*P.m. C889 (II)*	*C.f. Mantio (I)*
	I_{50}	*MIC*[a]	I_{50}	*MIC*[a]	I_{50}	*MIC*[a]	I_{50}	I_{50}
OH (clav. acid)	0.06	0.04	0.07	8	0.03	0.8	0.03	10
H	0.12	0.08	0.09	8	0.05	1.6	–	5
OCONHMe	1.5	0.6	2.5	16	2.5	3	–	0.45
OMe	0.05	0.02	0.18	8	0.07	0.6	0.01	8.5
OCH_2Ph	0.005	0.01	0.1	31	0.04	12.5	0.02	4.4
SMe	0.11	0.1	0.04	4	0.13	0.8	0.01	>>10[b]
$N(CH_2Ph)_2$	0.002	0.4	0.04	62.5	0.08	25	0.01	0.62
Ampicillin		500		>2000		1000		

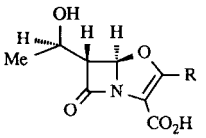
[a]MIC of ampicillin in presence of 5 μg/ml of inhibitor; [b]β-lactamase from *Enterobacter cloacae* P99; [c]classification of enzymes based upon Richmond-Sykes. For abbreviations, see footnote to *Table 6.1*.

β-lactamase inhibition [55]. It has a much greater potency than clavulanic acid against the cephalosporinase enzymes, but weaker activity against class III and class IV enzymes. It was also found to be unstable in solution [56]. More recently, Shionogi chemists have prepared oxapenem derivatives with the thienamycin-type hydroxyethyl side chain at C-6 [57]. The C-2 isopropyl derivative (33b) was found to be more stable than the C-2 methyl derivative (33a) (half lives at 37°C in pH 7.0 buffer 200 min and 43 min respectively) and displayed potent cephalosporinase inhibitory activity and some penicillinase inhibitory activity (*Table 6.6*).

PENICILLANIC ACID SULPHONE

Penicillanic acid sulphone (sulbactam; CP-45,899) (23) was shown to be a potent irreversible inhibitor of β-lactamases from classes II, III and IV. It is

Table 6.6. β-LACTAMASE INHIBITORY ACTIVITY OF OXAPENEMS [57]



(33)

(a) R = Me (b) R = $CHMe_2$

Compound	*Minimum effective concentration (μg/ml)*[a]			
	M.m SR7 (Ceph)	*E.cl. SR92 (Ceph)*	*K.o. SR696 (Pen)*	*E.co. TEM (Pen)*
(33a)	0.016	0.008	32	63
(33b)	0.008	0.016	>63	16
Clavulanic acid	>250	>250	0.063	0.063

[a]The minimum effective concentration was determined by a spot test using nitrocefin; Ceph, cephalosporinase; Pen, penicillinase. For abbreviations, see footnote to *Table 6.1*.

generally less potent than clavulanic acid except against class I β-lactamases, which are only poorly inhibited by both compounds. The comparative β-lactamase inhibitory activity of sulbactam and clavulanic acid is shown in *Table 6.7* [18].

Like clavulanic acid, sulbactam possesses only weak antibacterial activity but displays synergistic activity in combination with many penicillins and cephalosporins. Comparative synergistic activity of sulbactam and other β-lactamase inhibitors, in combination with ampicillin and amoxycillin, is presented in later sections.

By the oral and parenteral routes, clavulanic acid plus ampicillin was more effective (two to five-fold) than sulbactam plus ampicillin in protecting mice infected with *K. aerogenes, Pr. mirabilis* and *S. aureus* strains. Against organisms producing plasmid-mediated TEM β-lactamase, parenteral clavulanic acid at 1 to 5 mg/kg gave excellent results with ampicillin and cefazolin, whereas sulbactam at 100 mg/kg showed only marginal synergistic activity [58].

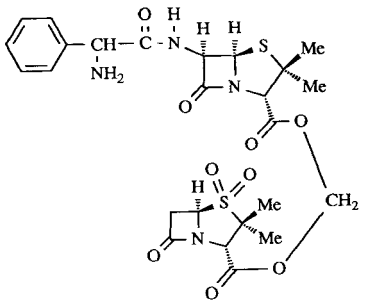
Sulbactam was poorly absorbed by animals and man by the oral route, though parenterally it gave levels similar to that of ampicillin and amoxycillin in the dog and rat [59]. A solution to the problem of poor

Table 6.7. β-LACTAMASE INHIBITORY ACTIVITY OF SULBACTAM, 6-β-HALOPENICILLANIC ACIDS AND 6-ACETYLMETHYLENE PENICILLANIC ACID COMPARED WITH CLAVULANIC ACID, WITH AND WITHOUT PRE-INCUBATION [18]

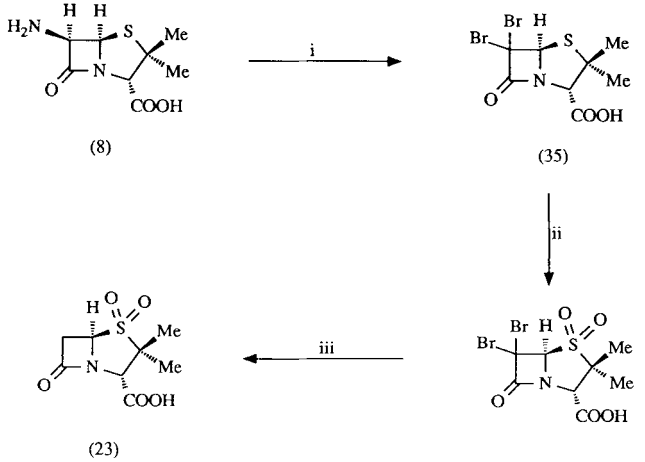
Organism	*I_{50} (μg/ml)[a] with (+) and without (−) 5 minute pre-incubation*									
	Sulbactam (23)		*6-β-Bromo-penicill-anic acid (24)*		*6-β-Iodo-penicill-anic acid (25)*		*6-Acetyl-methylene penicillanic acid (27)*		*Clavulanic acid (17)*	
	+	−	+	−	+	−	+	−	+	−
E.cl. P99 (I)[b]	5.8	>50	1.5	>50	5.5	>50	10	>50	110.0	>4000
P.m. C889 (II)	–	–	–	–	–	–	0.007	15.0	0.015	10.0
K.p. E70 (IV)	5.5	20	0.07	12.5	0.014	12.0	0.015	5.2	0.007	0.63
E.co. TEM-1 (III)	1.4	2.0	0.15	8.0	0.06	5.5	0.005	2.5	0.06	0.7
S.a. Russell	1.6	>50	1.5	50	0.7	>50	0.15	>50	0.03	75.0

[a]Concentration required to give 50% protection of 250 μg/ml nitrocefin substrate after a 5 min reaction time; [b]Enzyme classification based upon Richmond-Sykes. For abbreviations, see footnote to *Table 6.1.*

bioavailability was provided by the mutual pro-drug, sultamicillin (34), which is a double ester of formaldehyde hydrate whereby one of the hydroxyl groups has been esterified with ampicillin and the other with sulbactam. This resulted in high serum levels of sulbactam and ampicillin in a balanced proportion after oral administration [60].



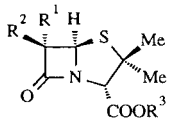
(34) Sultamicillin



Scheme 6.5. Synthesis of sulbactam [61].
Reagents: (i) $NaNO_2$, Br_2, MDC/2.5N H_2SO_4, 5°C; (ii) $KMnO_4$; (iii) H_2, Pd/C, EtOAc/aq. $NaHCO_3$.

Sulbactam was prepared from 6-APA (8) by the procedure shown in *Scheme 6.5* [61]. Modification of the diazotization-bromination procedure first reported by Clayton [62] provided 6,6-dibromopenicillanic acid (35) in high yield. Oxidation, followed by catalytic hydrogenation then provided sulbactam (23) in approximately 60% overall yield from 6-APA.

Penicillanic acid sulphones, such as sulbactam, are believed to inhibit β-lactamases by a similar mechanism to that of clavulanic acid (*Scheme 6.6*) [31, 37]. The initially formed acyl enzyme (38) is thought to partition



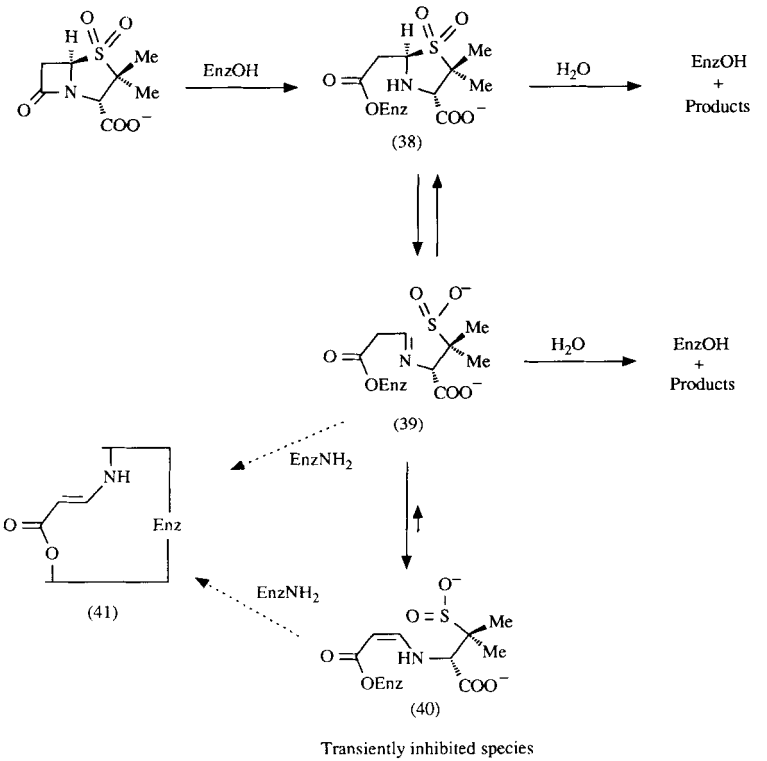
(24) $R^1 = H$, $R^2 = Br$, $R^3 = H$

(35) $R^1 = R^2 = Br$, $R^3 = H$

(36) $R^1 = Br$, $R^2 = R^3 = H$

(37) $R^1 = R^2 = Br$, $R^3 = SiMe_3$

between turnover and rearrangement into inert structures. The latter probably involves the opening of the thiazolidine ring to yield (39), followed by rearrangement into the hydrolytically more inert β-amino-acrylate system (40). Both intermediates (39) and (40) may then react further with basic functionalities within the enzyme, such as lysine functionalities, to yield inactivated species such as (41). A variety of penicillanic acid sulphones have been described and they are all believed to function in this manner. They do, however, have different β-lactamase specificities. The partitioning of (38) and (39) between the various pathways and thus the effectiveness of the inhibitor is a strong function of the inhibitor structure and the enzyme.



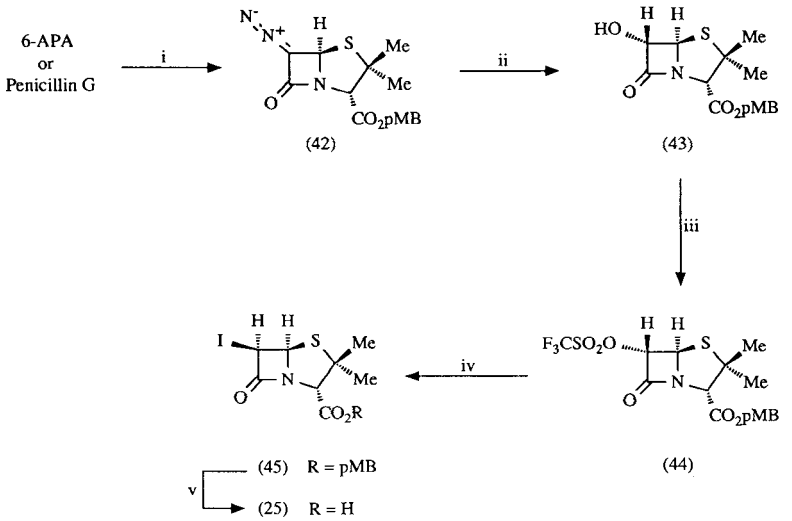
Scheme 6.6. Mechanism of action of sulbactam [31, 37].

6-β-HALOPENICILLANIC ACID DERIVATIVES

The discovery in 1978 that 6-β-bromopenicillanic acid is a potent inhibitor of bacterial β-lactamases [41] prompted a thorough investigation of the chemistry and biology of 6-β-halopenicillanic acids and their derivatives.

6-β-Bromopenicillanic acid (24) was originally obtained as a mixture with its inactive 6α-bromo epimer (36), either by aqueous equilibration of the latter at pH 9.1 and 30°C for 3 to 4 days, or by selective reduction of 6,6-dibromopenicillanic acid (34) [41]. The pure 6-β-bromopenicillanic acid (24) was subsequently obtained in pure form by separation of the epimeric mixture [42], as well as by the stereo-selective reduction of the trimethylsilyl 6,6-dibromopenicillanate (37) with tri-n-butyltin hydride, followed by hydrolysis and sodium salt formation [63].

The preparation of 6-β-iodopenicillanic acid (25) from 6-APA (8) was reported by Pfizer chemists (*Scheme 6.7*) [64]. The p-methoxybenzyl ester of 6-α-hydroxypenicillanic acid (43), prepared by the method of Sheehan *et al.* [65], was converted to the triflate (44), which upon reaction with sodium iodide gave the 6-β-iodopenicillanate ester (45); deprotection then provided the free acid (25).



Scheme 6.7. Preparation of 6-iodopenicillanic acid [64].
Reagents: (i) see [65]; (ii) $HClO_4$, aq. acetone; (iii) CF_3SO_2Cl, Et_3N; (iv) NaI, acetone; (v) TFA. *p*MB = *p*-methoxybenzyl

Table 6.8. SYNERGISTIC EFFECT OF 6-β-BROMOPENICILLANIC ACID (24) AND 6-β-IODOPENICILLANIC ACID (25) IN COMBINATION WITH AMPICILLIN AND CEFAZOLIN [66]

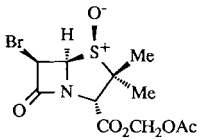
Organism	*MIC (μg/ml)*					
	Ampicillin alone	*Ampicillin MIC in combination with 5 μg/ml of:*		*Cefazolin alone*	*Cefazolin MIC in combination with 5 μg/ml of:*	
		(24)	*(25)*		*(24)*	*(25)*
C.d.	50	3.1	3.1	1.6	1.6	1.6
C.f.	100	25	25	>100	>100	>100
E.cl.	100	50	50	>100	>100	50
*E.co.*TEM	>200	3.1	3.1	12.5	1.6	1.6
K.p.	>200	12.5	12.5	12.5	1.6	1.6
Se.m.	>200	>200	>200	>100	>100	>100
S.a.	>200	0.4	1.6	25	25	6.3

For abbreviations, see footnote to *Table 6.1.*

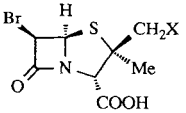
Both 6-β-bromopenicillanic acid (24) and 6-β-iodopenicillanic acid (25) were as potent as clavulanic acid and inhibited the same range of β-lactamase enzymes (*Table 6.7*) [18, 43, 66]. They acted synergistically with ampicillin and cefazolin to inhibit β-lactamase containing *S. aureus*, those members of the *Enterobacteriaceae* which contain plasmid β-lactamases such as the TEM enzyme, and a *Klebsiella* spp. which contains a chromosomal β-lactamase. They did not however inhibit most of the β-lactamases of organisms such as *Enterobacter* spp. *Providencia* spp., *P. aeruginosa*, or *Serratia* spp. (*Table 6.8*) [66].

von Daehne and co-workers [67] also described the synthesis of a series of 6-β-bromopenams (47), substituted in the 2-β-methyl position, from the acetoxymethyl ester of 6-β-bromopenicillanic acid (R)-sulphoxide (46) by chemistry which will be discussed in more detail in later sections. In general, the various penams (47) were less active than the parent compound (24).

6-β-Bromopenicillanic acid (24) is a powerful, irreversible, active-site-directed inhibitor of β-lactamases [41]. Its mechanism of action has been studied extensively. Pratt and Loosemore [41] reported that the β-lactamase I of *Bacillus cereus* was extremely susceptible to inhibition by 6-β-bromopenicillanic acid, being completely inhibited at less than micromolar concentrations through what was probably a 1:1 interaction. This was confirmed by radiolabelling studies using [^{3}H] bromo-penicillanic acid; the



(46)



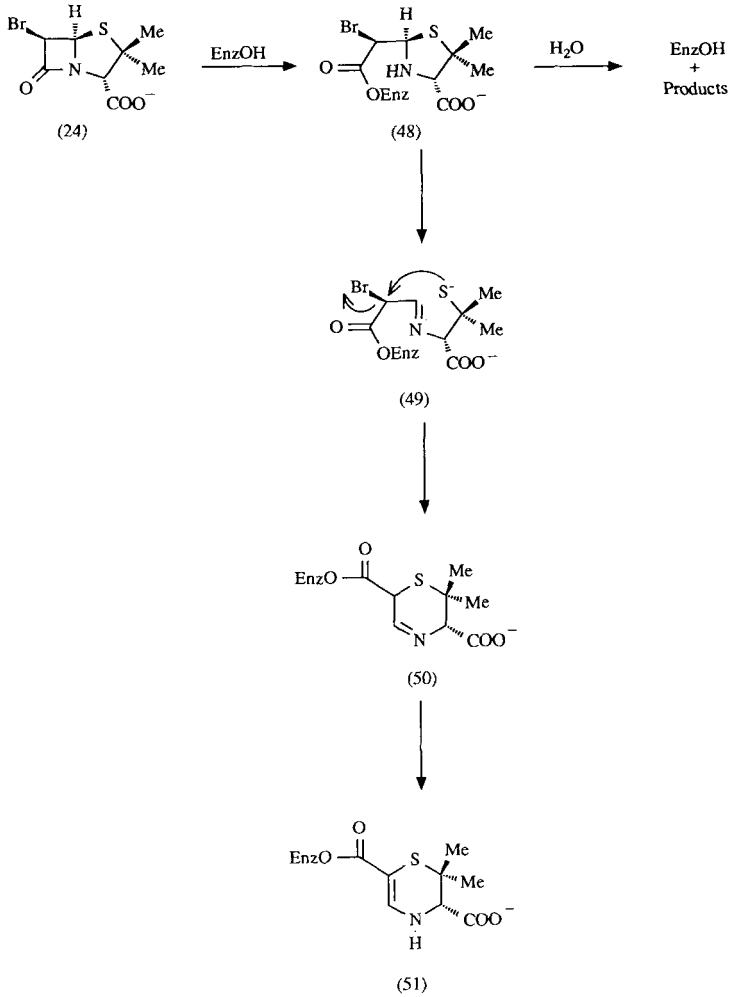
(47) X = F, N_3, OAc, OMe, OEt, SCN

label was reported to be bound to serine 70, thus confirming that β-lactamases function as serine proteases which form covalent acyl-enzyme intermediates [68, 69]. Inactivation of the *B. cereus* enzyme was accompanied by a new chromophore at 326 nm, which is believed to be due to an enzyme-bound dihydrothiazine (51). On this basis it was postulated that the mechanism of action of 6-β-bromopenicillanic acid involved the nucleophilic displacement of bromide from the C-6 position by the transiently formed thiolate (49) to form the stable dihydrothiazine (51) [70-72]. This acyl-enzyme species was found to be virtually inert and released the radiolabel only after treatment with alkali [67] (*Scheme 6.8*).

6-ACETYLMETHYLENE PENICILLANIC ACID

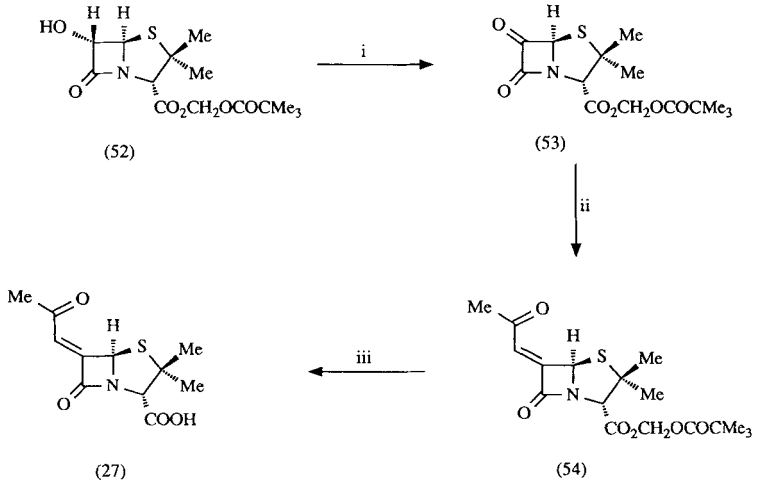
Hoffman-La Roche's contribution to the design of semi-synthetic β-lactamase inhibitors was the synthesis of 6-(*Z*)-acetylmethylenepenicillanic acid (Ro 15-1903) (27) from the 6-α-hydroxypenicillanate ester (52) (*Scheme 6.9*) [73]. Oxidation of alcohol (52) with dimethyl sulphoxide-trifluoroacetic anhydride gave the ketone (53) which in turn reacted with 1-triphenylphosphoranylidene-2-propanone to yield the (*Z*)-isomer (54) of the acetonylidenepenicillanate as the major product. Deprotection then provided 6-acetylmethylenepenicillanic acid (27).

6-Acetylmethylenepenicillanic acid (27) has been shown to be a powerful, broad spectrum irreversible β-lactamase inhibitor. It inhibits a wide range



Scheme 6.8. Mechanism of action of 6-β-bromopenicillanic acid [70].

of chromosomally and plasmid-mediated β-lactamases and protects β-lactamase-labile penicillins and cephalosporins from hydrolysis [74–76]. Against isolated enzymes it is generally more potent than either clavulanic acid or sulbactam (*Table 6.7*). However, this *in vitro* advantage against clavulanic acid and sulbactam is lost *in vivo*, due to lack of stability.



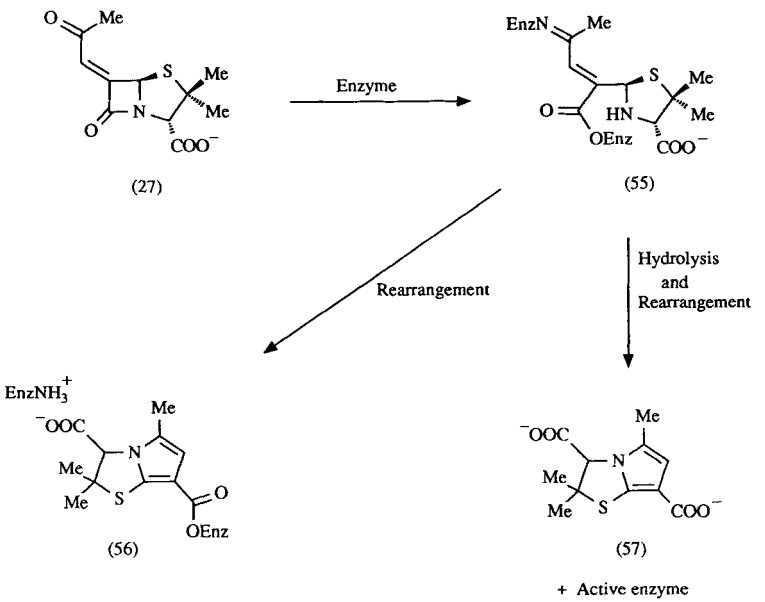
Scheme 6.9. Preparation of 6-(Z)-acetylmethylene penicillanic acid [73].
Reagents: (i) DMSO-$(CF_3CO)_2O$, −65°C; (ii) Ph_3P=CHCOMe; (iii) hog liver esterase.

Attempts were therefore made to prepare pro-drug forms of (27) which might improve chemical stability, but retain site-specific delivery. This approach met with limited success [77].

Based upon studies involving the interaction of 6-acetylmethylenepenicillanic acid (27) with TEM-1 β-lactamase, as well as its chemical reactivity with sodium methoxide and hydroxylamine, Arisawa and Adam [45] proposed a mechanism for the inhibition of TEM-1 β-lactamase by (27) (*Scheme 6.10*). They proposed the initial formation of a rather stable inactivated enzyme (55). This was followed by either slow hydrolysis of the acyl bond, releasing active enzyme, or rearrangement to (56). The stability of this intermediate was explained on the grounds of its pyrrolic structure; the conjugated ester behaves as a vinylogous urethane, which is known to be resistant to hydrolysis [72].

6-METHOXYMETHYLENE PENICILLANIC ACID

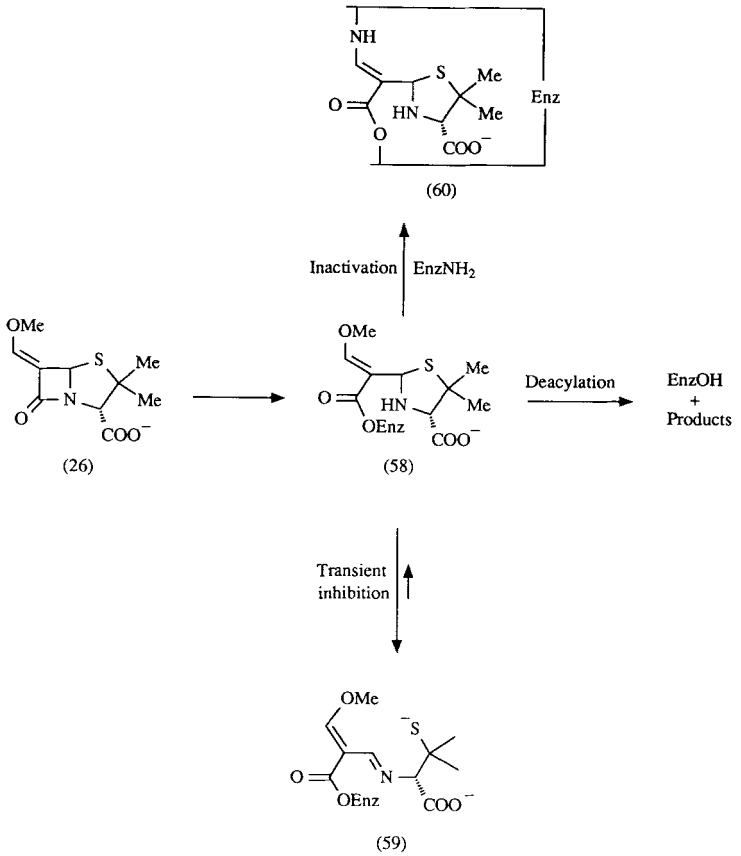
6-Methoxymethylene penicillanic acid (26) was designed by Brenner and Knowles [78] as a potential inhibitor for which the first-formed acyl-enzyme intermediate would be a relatively stable vinylogous ester (58) (*Scheme 6.11*) possessing a heteroatom in the β-position. It was envisaged that this intermediate would resemble the transiently inhibited β-aminoacrylate



Scheme 6.10. Mechanism of action of 6-acetylmethylene penicillanic acid [45].

species which is responsible for the activity of clavulanic acid (*Scheme 6.4*) and sulbactam (*Scheme 6.6*). Furthermore, after reaction with the enzyme, the potential exists for irreversible inactivation of the enzyme by Michael addition of an enzyme nucleophile to the α,β-unsaturated ester, followed by the expulsion of methanol to yield a β-aminoacrylate. Indeed, the interaction of this novel penam with the TEM-2 β-lactamase from *E. coli* was studied and shown to be a potent, irreversible inhibitor of this enzyme. Its speculated mode of action is shown in *Scheme 6.11*. Nucleophilic attack by the active-site serine-70 on the β-lactam carbonyl results in acylation of the enzyme, which may then partition in one of three ways: (i) deacylation of (58) to regenerate active enzyme, (ii) rearrangement to the transiently inhibited form (59), or (iii) further reaction with enzymic amine to yield the irreversibly inactivated enzyme (60). All eventualities are consistent with experimental data. To date, no synergy data have been reported.

6-Methoxymethylene penicillanic acid (26) was prepared from benzyl 6-oxopenicillanate (61) by treatment with the anion of methoxy-(trimethylsilyl)methane. Acylation of the resulting alcohols, (62) and (63), followed by hydrogenolysis provided the acids, (66) and (67), which upon

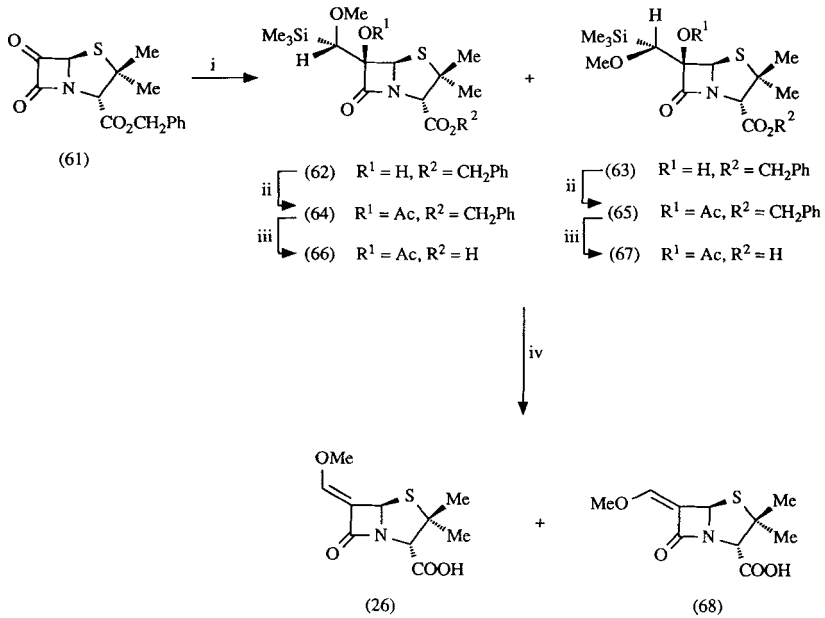


Scheme 6.11. Hypothetical mechanism of action of 6-methoxymethylene penicillanic acid. Reprinted with permission from Brenner and Knowles [78]. Copyright (1984) American Chemical Society.

fluoride-promoted elimination yielded an equilibrium mixture of the (*Z*)-and (*E*)-isomers of the methoxymethylene penam, (26) and (68), respectively (*Scheme 6.12*) [44].

6-HETEROCYCLYLMETHYLENE PENAM SULPHONES

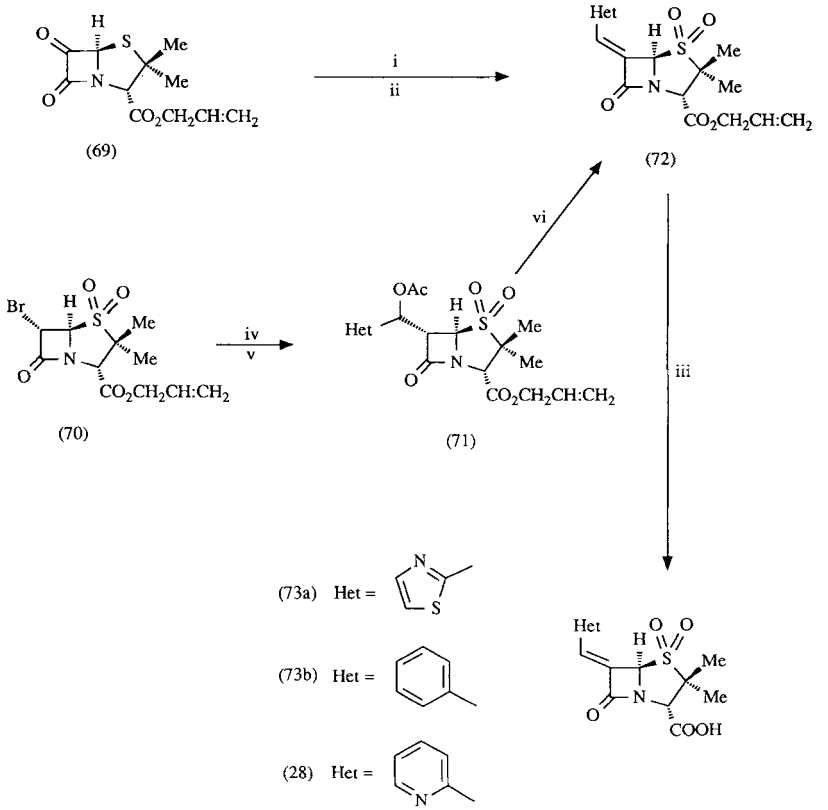
In 1987, Pfizer chemists reported the synthesis of a series of 6-heterocyclylmethylene penam sulphones (73) from either allyl 6-oxopenicil-



Scheme 6.12. Synthesis of 6-(methoxymethylene) penicillanic acid [44].
Reagents: (i) $LiCH(OMe)SiMe_3$, −100°C, THF; (ii) Ac_2O, Et_3N, 4-DMAP (cat.); (iii) H_2, Pd/C; (iv) CsF, DMSO, 80°C, 2h.

lanate (69), or allyl 1,1-dioxo-6α-bromopenicillanate (70) (*Scheme 6.13*) [79]. Reaction of (69) with the heterocyclylphosphorane, followed by oxidation and deprotection provided predominantly the (*Z*)-isomer (73). Alternatively, treatment of the 6α-bromo-derivative (70) with methyl magnesium bromide, followed by the heterocyclic aldehyde and acetic anhydride gave the isomeric acetates (71). Base-catalysed elimination and deprotection then gave the (*Z*)-isomer of the 6-heterocyclylmethylene penam sulphone (73), accompanied by varying amounts of the less active (*E*)-isomer.

The (*Z*)-6-heterocyclylmethylene penam sulphones were shown to be effective β-lactamase inhibitors and potent ampicillin and cefazolin potentiators against both Gram-positive and Gram-negative β-lactamase producing bacteria [79]. In particular, several of these analogues having a π-deficient 2-heteroaryl substituent attached to the C-6 methylene positon, such as the 6-(2-pyridyl)methylene derivative (28), displayed exceptional activity. In combination with ampicillin or cefazolin they exhibited



Scheme 6.13. Preparation of 6-heterocyclylmethylene penam sulphones [46, 79].
Reagents: (i) Ph_3P=CHHet, THF, −78°C; (ii) m-chloroperbenzoic acid; (iii) tetrakis-(triphenylphosphine)palladium, PPh_3, potassium 2-ethylhexanoate, EtOAc, 30 min, r.t.; (iv) a) MeMgBr, −78°C; b) HetCHO, −78°C; (v) Ac_2O, pyridine; (vi) 1,5-diazabicyclo[4.3.0]non-5-ene.

synergistic activity at least equal to clavulanic acid, 6-β-bromopenicillanic acid, sulbactam or the 6-acetylmethylene penam against β-lactamase producing strains (*Table 6.9*).

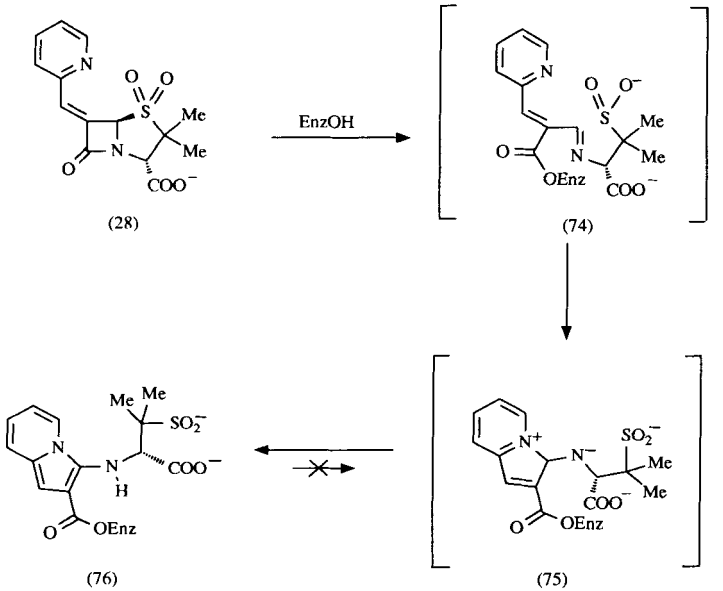
A mechanism of action for the π-deficient 2-heteroaryl derivatives was postulated based upon the reaction of 6-(2-pyridyl)methylene penicillanic acid sulphone (28) with sodium methoxide (*Scheme 6.14*) [46]. Chen and co-workers proposed that after bimolecular interaction between the enzyme and (28), an aromatic acyl-enzyme ester (76) is obtained. This conjugated

Table 6.9. SYNERGISTIC ACTIVITY OF 6-HETEROCYCLYLMETHYLENE PENAM SULPHONES WITH AMPICILLIN [79]

Compound	*MIC[a] (μg/ml) of a 1:1 combination of inhibitor with ampicillin*				
	S.a. 01A400 (Pen)	*E.co. 51A129 (Pen)*	*K.p. 53A079 (Pen)*	*E.cl. 67B009 (Ceph)*	*M.m. 97A001 (Ceph)*
(28)	0.39	25	6.25	25	3.12
(73a)	0.39	50	3.12	100	3.12
(73b)	25	>100	50	>100	25
Clavulanic acid	0.39	12.5	12.5	100	>100
Ampicillin alone	50	>200	100	200	>200

[a]Concentration of both compounds; Pen: penicillinase; Ceph: cephalosporinase. For abbreviations, see footnote to *Table 6.1.*

acyl-enzyme ester, having an electron-donating amino group at the ortho-position is believed to be resistant to hydrolysis, thus accounting for

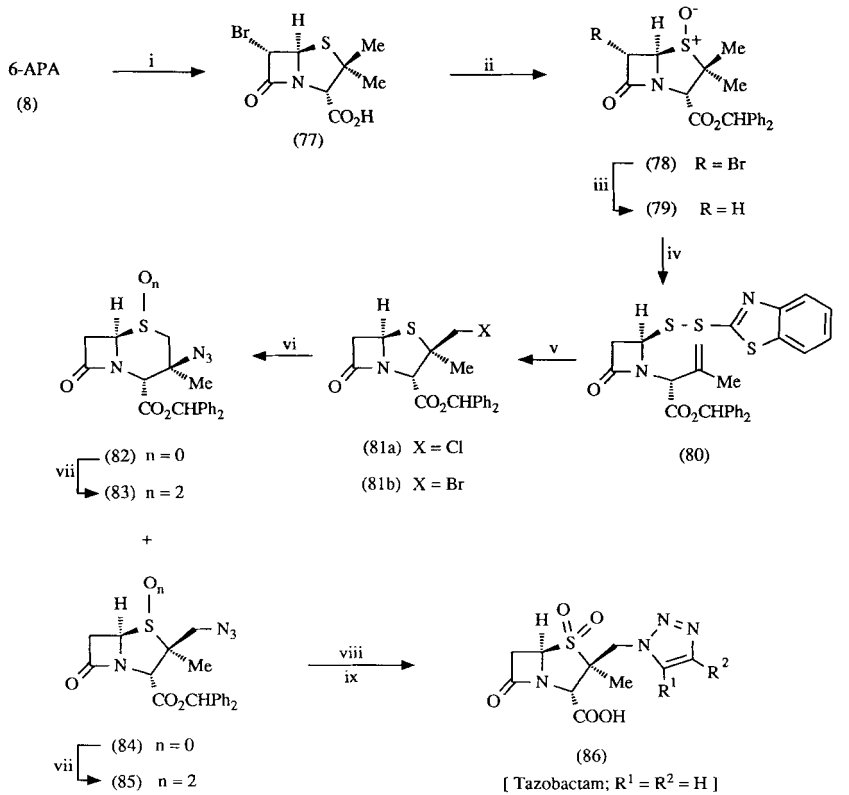


Scheme 6.14. Proposed mechanism of action of 6-(2-pyridyl)methylene penicillanic acid sulphone. [46]

the potent, irreversible inhibition observed. Support for the proposed mechanism of action was provided by the inferior activity of the phenyl analogue (73b) (*Table 6.9*).

2-β-(SUBSTITUTED METHYL) PENAM SULPHONES

The discovery of the β-lactamase inhibitory activity of sulbactam signalled a resurgence of interest in the nuclear modification of 6-APA (8). Micetich and co-workers concentrated their efforts on the synthesis of 2-β-



Scheme 6.15. Preparation of tazobactam and analogues [48–50, 82].
Reagents: (i) $NaNO_2$, HBr; (ii) a) $MeCO_3H$; b) $MeCO_3H$, $Ph_2C{=}N.NH_2$, H_2SO_4; (iii) Zn, AcOH; (iv) 2-mercaptobenzothiazole, toluene, reflux; (v) CuX_2, MDC, r.t. or 0°C; (vi) NaN_3, DMF, H_2O; (vii) $KMnO_4$; (viii) substituted acetylene; (ix) H_2, Pd/C [for R^1=H, R^2=$SiMe_3$, (x) a) KF, 18-crown-6, DMF; b) H_2, Pd/C].

(substituted methyl) penicillanic acid sulphone derivatives and the effect that this substitution had on the β-lactamase inhibitory activity. Research in this area resulted in the identification of tazobactam (YTR-830) (30), a potent inhibitor of bacterial β-lactamases [48, 49]; when combined with amoxycillin, tazobactam displayed a synergistic effect which was superior to that of sulbactam [50, 80, 81].

The synthesis of tazobactam from 6-APA (*Scheme 6.15*) proceeded *via* the 2-β-(chloromethyl)penam ester (81a), which was first prepared by Gottstein and co-workers [47] during the synthesis of 2-β-(chloromethyl)-2-α-methylpenam-3α-carboxylic acid 1,1-dioxide (29). 6-APA (8) was converted to 6α-bromopenicillanic acid (77) by treatment with sodium nitrite and hydrobromic acid. Oxidation with peracetic acid in the presence of benzophenone hydrazone gave benzhydryl 6α-bromopenicillanate-1-oxide (78) and reduction with zinc and acetic acid gave benzhydryl penicillanate-1-oxide (79). The unsymmetrical azetidinone disulphide (80) was obtained by heating with 2-mercaptobenzothiazole; reaction with copper (II) chloride

Table 6.10. BIOLOGICAL ACTIVITY OF YTR CLASS OF COMPOUNDS [48, 83]

Compounds	I_{50} (μg/ml)	*Ampicillin MIC (μg/ml) in presence of 10 μg/ml of inhibitor*			
	Bacillus sp.[a]	*S.a. S-54K*	*E.co. TH-13*	*P.m. 121K*	*Se.m. TH-5*
(86a) R^1=R^2=COOMe	0.3	0.39	25	1.56	100
(86b) R^1=H, R^2=COOMe	0.049	0.2	3.13	0.78	12.5
(86c) R^1=COOMe, R^2=H	0.3	0.2	6.25	0.78	25
(86d) R^1=H, R^2=COOK	0.6	0.2	6.25	0.78	6.25
(86e) R^1=H, R^2=NH_2	0.7	–	–	–	–
(86f) R^1=H, R^2=Ph	0.04	–	–	–	–
Tazobactam	0.69	0.2	3.13	1.56	3.13
Sulbactam	10	0.2	6.25	6.25	3.13
Amp alone	–	25	400	>400	400

[a]Substrate, penicillin G. For abbreviations, see footnote to *Table 6.1*.

or bromide then gave the 2-β-halomethyl penams (81). Treatment of either 2-β-halo-derivative with sodium azide gave a 3:2 mixture of the 2-β-azidomethylpenam (84) and the 3-β-azidocepham (82), which was separated by fractional crystallisation after oxidation to the respective sulphones (85) and (83) [82].

On heating the 2-β-azidomethyl penam sulphone (85) with suitable acetylenes, a series of 2-β-(1,2,3-triazolyl)methyl penam sulphone esters was obtained, which upon deprotection gave the free acids (86) [48, 49]. In particular, reaction of (85) with either vinyl acetate or (trimethylsilyl)acetylene provided the parent triazole, tazobactam (30), after hydrogenation (and prior potassium fluoride–18-crown-6 treatment in the case of the TMS acetylene) [50].

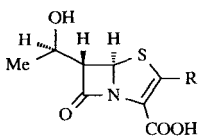
The β-lactamase inhibitory activity of some representative examples of the 'YTR class' of compounds is shown in *Table 6.10* [48, 83]. A comparison of the *in vitro* and *in vivo* activity of tazobactam with other inhibitors [*Tables 6.11, 6.12, 6.14–6.18*] shows that it has similar potency to clavulanic acid but is significantly inferior to BRL 42715 (31).

The mechanism of action of tazobactam is believed to be similar to that discussed earlier for sulbactam.

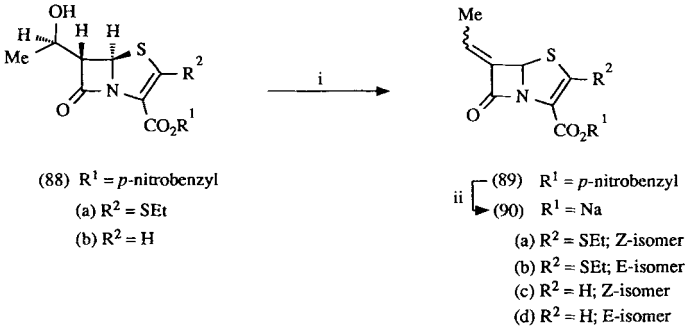
6-(SUBSTITUTED METHYLENE) PENEMS

The penems were conceived by R.B. Woodward in 1976 as structural hybrids of penicillins and cephalosporins [84]. Unlike the carbapenems and clavams, they are not found in nature. They do however possess important biological properties and have therefore created considerable interest in both their total synthesis and their biological evaluation [85].

Whilst most attention focused upon the antibacterially active 6-(1-hydroxyethyl) penems (87), Osborne (SmithKline Beecham) found in 1981 that the dehydration of such penems (*Scheme 6.16*) provided compounds which had only weak antibacterial activity, but good β-lactamase inhibitory activity [86, 87]. Their spectrum of β-lactamase inhibitory activity is broader than that of clavulanic acid and they have greater potency than sulbactam.



(87) R = alkyl, S-alkyl, etc.



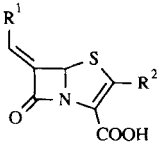
Scheme 6.16. Preparation of 6-ethylidene penems [86, 87].
Reagents: (i) PPh_3, diethyl azodicarboxylate, THF; (ii) a) H_2, Pd/C, aq. 1,4-dioxan; b) $NaHCO_3$.

The β-lactamase inhibitory activity of the 6-ethylidenepenems, in comparison with potassium clavulanate and sulbactam is shown in *Table 6.11*. It can be seen that both (*Z*)- and (*E*)- isomers of the penems inhibited all four β-lactamases, including the class Ia enzyme of *Enterobacter cloacae*,

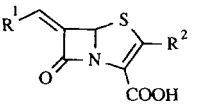
Table 6.11 BIOLOGICAL ACTIVITY OF 6-ETHYLIDENEPENEMS [87]

Compound	*I_{50} (μg/ml)*[a,b]				*Antibiotic MIC (μg/ml) in presence of inhibitor*[c]			
	E.cl. (Ia)[d]	*E.co. TEM (III)*	*K.p. (IV)*	*S.a. Russell*	*Cephaloridine*	*Amoxycillin*		
					E.cl. (Ia)	*E.co. TEM (III)*	*K.p. (IV)*	*S.a. Russell*
(90a)	0.6	0.007	0.001	0.13	2.5	5.0	2.5	0.5
(90b)	0.4	0.15	0.03	2.5	2.5	5.0	2.5	0.5
(90c)	1.0	0.04	0.015	0.6	10	5.0	2.5	1.0
(90d)	0.4	0.2	0.07	3.2	–	–	–	–
Clavulanic acid	>50	0.06	0.03	0.08	1000	5.0	5.0	0.2
Sulbactam	4.0	1.0	6.0	1.3	–	–	–	–
Inhibitor alone					1000	>1000	500	100

[a]Substrate, nitrocefin; [b]after 5 min pre-incubation; [c]concentration of inhibitor, 5.0 μg/ml for Gram-negative bacteria and 1.0 μg/ml for *S.a.*; [d]Enzyme classification based on Richmond-Sykes. For abbreviations, see footnote to *Table 6.1*.



(91)



(92)

(a) R^1 =  , $R^2 = H$

(b) R^1 =  , $R^2 = H$

(c) R^1 =  , $R^2 = H$

(d) R^1 =  , $R^2 = H$

(e) R^1 =  , R^2 = SEt

which is only weakly inhibited by potassium clavulanate. Sulbactam showed similar broad-spectrum inhibitory activity but was much less potent than the penems. The inhibitory activity of these compounds was reflected also in the synergy observed with β-lactams against β-lactamase producing organisms (*Table 6.11*). Whilst they were as effective as potassium clavulanate in reducing the MIC values of amoxycillin against *K. pneumoniae* and resistant strains of *E. coli* and *S. aureus*, they also improved the activity of amoxycillin against *E. cloacae*. Potassium clavulanate had no effect against this organism. The cephalosporinase activity of the penems was seen to a greater extent when tested in

Table 6.12. SYNERGISTIC AND β-LACTAMASE INHIBITORY ACTIVITY OF 6-HETEROCYCLYLMETHYLENE PENEMS [88]

Compound	I_{50}[a] *(μg/ml)*				*Antibiotic MICs (μg/ml) in the presence 1 μg/ml inhibitor*			
	E.cl (I)[b]	*E.co. (III)*	*K.p. (IV)*	*S.a.*	*Cepahaloridine*	*Amoxycillin*		
					E.cl. (I)	*E.co. (III)*	*K.p. (IV)*	*S.a.*
(90c)	1.000	0.040	0.015	0.600	256	4	2	1.0
(91a)	0.020	0.008	0.028	0.400	8	4	32	0.5
(92a)	3.000	3.500	2.200	In	256	128	>128	>8.0
(91b)	0.003	0.002	0.003	0.021	16	2	4	0.3
(91c)	0.005	0.003	0.002	0.013	16	2	8	0.3
(92c)	0.400	0.350	0.150	5.500	32	8	4	2
(91d)	0.005	0.001	0.003	0.045	8	2	16	0.5
(91e)	0.003	0.130	0.040	0.012	128	16	64	0.3
Clav. acid	In	0.050	0.019	0.060	>256	2	2	0.5
Sulbactam	2.8	1.90	10.0	1.50	256	128	32	4.0
Tazobactam	0.02	0.02	0.10	0.35	128	16	16	1.0
No inhibitor	–	–	–	–	>256	>256	256	128.0

[a]Concentration giving 50% inhibition of the rate of hydrolysis of nitrocefin after pre-incubation of enzyme and inhibitor for 5 min; [b]enzyme classification based on Richmond-Sykes; In, inactive at 10 μg/ml. For abbreviations, see footnote to *Table 6.1*.

combination with cephaloridine, when they reduced the MIC values against all the test organisms other than *P. aeruginosa* [87].

It can also be seen from the data that neither changes in the geometry of the ethylidene double bond nor of the C-2 substituent resulted in any gross changes of synergistic activity.

Further studies by Osborne and co-workers showed the effect that replacement of the C-8 methyl group with other functions, including heterocyclyl groups, had on β-lactamase inhibitory activity. Of the first series of compounds to be reported, it was demonstrated that replacement of the C-8 methyl group in 6-ethylidenepenems (90) with either a thiophene or furan ring resulted in a considerable increase in synergistic activity (*Table 6.12*) [88]. The (*Z*)-isomers proved to be better inhibitors than the

Table 6.13. *IN VIVO* SYNERGISTIC ACTIVITY OF 6-HETEROCYCLYLMETHYLENE PENEMS [88]

	Antibiotic CD_{50}[a] as mg/kg × 3	
	E.co. TEM-1 (III)[c]	*E.cl. (I)*[c]
Antibiotic	Amoxycillin	Cefazolin
Inhibitor dose (mg/kg)[b]	5	2
Antibiotic alone	1000	200
Antibiotic + (91a)	200	100
Antibiotic + (91b)	200	200
Antibiotic + (91c)	63	60
Antibiotic + (91d)	200	175
Antibiotic + Clavulanic acid	2	–

[a]CD_{50}, curative dose for 50% of mice following dosing subcutaneously; [b]all β-lactamase inhibitors were inactive when dosed alone at the dose level used in the combination; [c]enzyme classification based upon Richmond-Sykes. For abbreviations, see footnote to *Table 6.1*.

corresponding (*E*)-isomers and the introduction of substituents in the C-2 position resulted in reduced inhibitory activity, particularly against *E. coli* TEM-1 and *K. pneumoniae* β-lactamases. The synergistic activity of the C-2 substituted derivatives was also reduced relative to that of the C-2 unsubstituted (*Z*)-isomers. Although the C-2 unsubstituted (*Z*)-isomers (91a–d) displayed potent *in vitro* β-lactamase inhibitory activity, they were still inferior to clavulanic acid *in vivo* against *E. coli* TEM-1 (*Table 6.13*). However, further studies on the effect of varying the heterocyclic substitutent identified a series of triazolylmethylene penem derivatives which displayed far superior *in vivo* as well as *in vitro* β-lactamase inhibitory activity when compared with clavulanic acid, sulbactam and tazobactam. The most active member of this series, (5R)-(*Z*)-6-(1-methyl-1,2,3-triazol-4-ylmethylene)penem-3-carboxylic acid (31) (BRL 42715) [89] is a potent inhibitor of a broad range of bacterial β-lactamases, including the plasmid-mediated TEM, SHV, OXA, and *staphylococcal* enzymes, as well as the chromosomally-mediated enzymes of *Bacteroides, Enterobacter, Citrobacter, Serratia, Morganella, Escherichia, Klebsiella*, and *Proteus* species.

Of the many derivatives prepared, BRL 42715 (31) was the compound with the best overall activity and stability. The enzyme, renal dehydropeptidase I (RDHP) is known to be a major cause of metabolic inactivation of carbapenems, such as imipenem, and penems. Whereas the ethylidene derivative (90c) showed substantial degradation of the biologically active (5*R*)-enantiomer in the presence of human kidney homogenate, BRL 42715 proved particularly stable, with 68% surviving after 1 hour's exposure to human kidney [90, 91]. In addition, BRL 42715 was only moderately bound (68%) to human serum.

The β-lactamase inhibitory activity of BRL 42715, in comparison with clavulanic acid, sulbactam and tazobactam is shown in *Table 6.14*. It can be seen that BRL 42715 displays potent and progressive broad spectrum β-lactamase inhibitory activity which represents a significant improvement over that of clavulanic acid, sulbactam and tazobactam. This was most noticeable with the chromosomal class I cephalosporinases, against which clavulanic acid is poorly active and sulbactam and tazobactam show only moderate activity.

In combination with amoxycillin, potent synergistic activity is seen

Table 6.14. INTRINSIC β-LACTAMASE INHIBITORY ACTIVITY OF BRL 42715 (31) COMPARED WITH THAT OF OTHER β-LACTAMASE INHIBITORS [92]

Organism[a]	*I_{50} values (μg/ml) with (+) and without (−) 5 min pre-incubation*							
	BRL 42715		*Clavulanic acid*		*Sulbactam*		*Tazobactam*	
	−	+	−	+	−	+	−	+
E.cl P99 (Ia)[b]	0.069	0.002	>50	>50	>50	5.0	>50	0.93
P.v. H (Ic)	0.009	0.003	0.84	0.017	1.8	0.12	0.32	0.006
P.m. C889 (II)	1.4	0.009	3.6	0.021	2.9	0.057	1.0	0.006
E.co. JT4 (TEM-I) (III)	0.044	0.002	0.88	0.055	3.0	1.7	0.12	0.028
K.p. E70 (IV)	0.036	0.001	1.0	0.011	15.7	3.8	0.68	0.047
E.co. (OXA-1) (V)	0.29	0.001	>50	0.71	>50	2.2	>50	1.1
E.co. (PSE-4) (V)	12.5	0.13	2.0	0.022	3.6	0.29	0.42	0.025
S.a. NCTC 11561	3.3	0.016	>50	0.063	>50	1.4	>50	0.27

[a]Enzymes in parentheses were produced particularly by the strains shown; [b]enzyme classification bases upon Richmond-Sykes system. For abbreviations, see footnote to *Table 6.1*.

against a wide range of β-lactamase producing organisms (*Table 6.15*). BRL 42715 markedly improved the activity of amoxycillin in agar-dilution tests against β-lactamase producing strains of *C. freundii, E. coli, E. aerogenes, K. pneumoniae* and *S. aureus*. Furthermore, it enhanced the activity of amoxycillin against a typical strain of *P. aeruginosa* at a higher concentration [92]. The protective effect of BRL 42715 was not restricted to amoxycillin. Both cefotaxime-susceptible and cefotaxime-resistant class I β-lactamase-producing strains were resistant to cefazolin alone but susceptible to cefazolin plus BRL 42715. Likewise, piperacillin, cefotaxime and ceftazidime were all ineffective against high-level class I β-lactamase-producing strains when tested alone, but when tested in combination with

Table 6.15. COMPARATIVE SYNERGISTIC ACTIVITIES OF BRL 42715 (31), TAZOBACTAM (30), CLAVULANIC ACID (17) AND SULBACTAM (23) WITH A RANGE OF β-LACTAMASE PRODUCING BACTERIA [92, 93]

Compound combination	*Concn. of inhibitor (μg/ml)*	*MIC (μg/ml)*							
		C.f. T1739[a] (I)[e]	*E.ae. 53[a] (I)*	*E.cl. P99[a] (Ia)*	*E.c. AmpC (high) (Ib)*	*E.c. TEM (high) (III)*	*E.c. OXA-1 (V)*	*K.p. TEM-1 (IVI III)*	*S.a.[b,c]*
Amoxycillin alone		>512	>512	>512	512	>512	>512	>512	>128
Amox + Sulbactam	16	–	–	–	128	>512	32	64	–
Amox + Clav. acid	4	–	–	–	256	32	32	4	2[d]
Amox + Tazobactam	4	512	>512	>512	256	64	64	8	–
Amox + BRL42715	1	1	2	64	2	4	2	1	0.12[d]
Piperacillin alone		512	>512	>512					
Pip + Tazobactam	4	128	512	512					
Pip + BRL 42715	1	4	16	2					
Cefazolin alone		>512	>512	>512					
Cef + Tazobactam	4	>512	>512	>512					
Cef + BRL 42715	1	4	2	16					
Cefotaxime		64	64	>64					
Cftm + Tazobactam	4	32	>64	64					
Cftm + BRL 42715	1	0.5	2	2					

[a]Cefotaxime resistant; [b]methicillin sensitive strains; [c]*MIC* for 50% of strains (21) tested; [d]inhibitor concentration, 0.2 μg/ml; [e]enzyme classification according to Richmond and Sykes. For abbreviations, see footnote to *Table 6.1*.

Table 6.16. EFFICACY OF AMOXYCILLIN ALONE AND IN COMBINATION WITH β-LACTAMASE INHIBITORS [93]

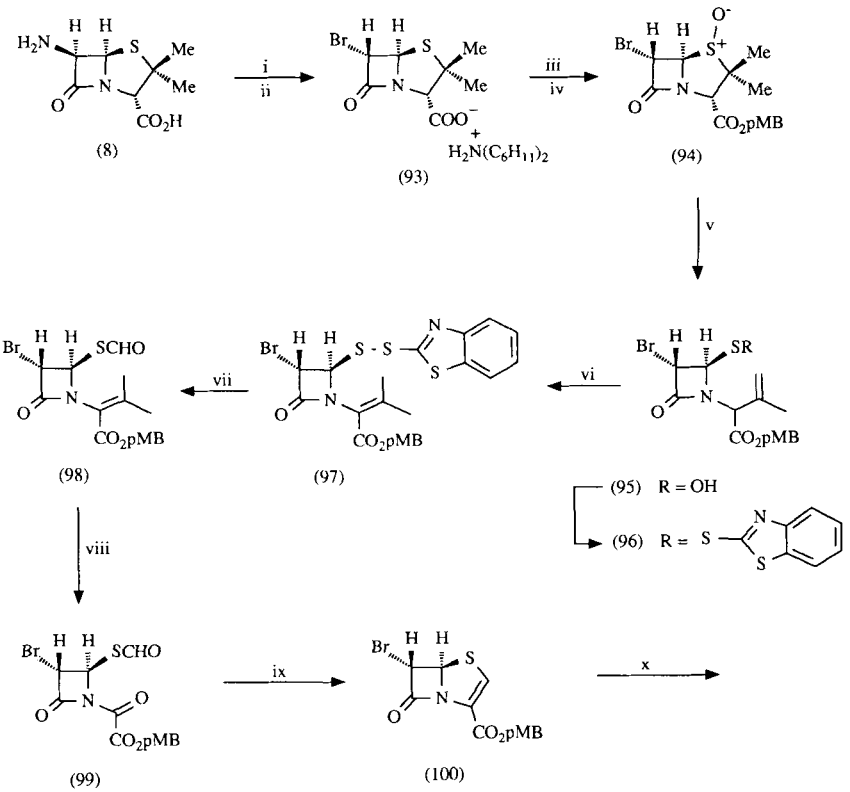
Inhibitor	*Amoxycillin CD_{50} (mg/kg) in combination with inhibitor*[a]					
	S.m. (Ia)[b]	*E.co.* AmpC (high) (Ib)	*P.v. (Ic)*	*Ps.a. (Id)*	*E.co. TEM-1 (high) (III)*	*E.co. (V)*
No inhibitor	40	>100	500	>1000	>1000	>1000
BRL 42715	5	4	4.2	20	22	8.5
Clavulanic acid	40	>200	10	>200	>200	>200
Tazobactam	30	>200	9.5	>200	>200	>200
Sulbactam	40	>200	45	>200	>200	>200
Inhibitor dosage	2 mg/kg × 2	2 mg/kg × 2	2mg/kg × 2	10 mg/kg × 3	2 mg/kg × 2	10 mg/kg × 3

[a]The inhibitors at doses of 2 or 10 mg/kg were mixed with varying doses of amoxycillin and administered subcutaneously to groups of 5 mice at time intervals of 1 and 5 hours or 1,3 and 5 hours post infection; [b]enzyme classification based upon Richmond-Sykes system. For abbreviations, see footnote to *Table 6.1*.

1 μg/ml BRL 42715, the MIC values obtained were generally much closer to those achieved against cefotaxime-susceptible strains [92].

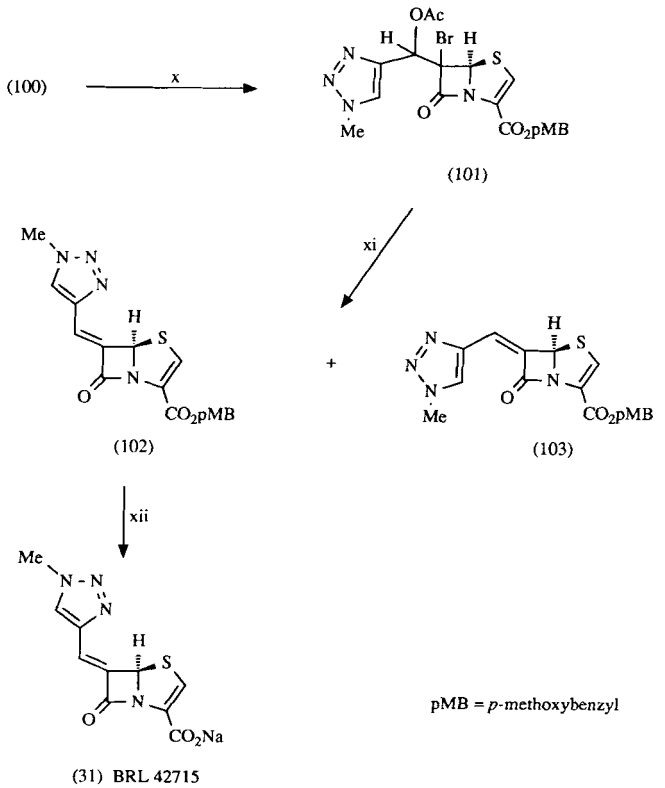
When given in conjunction with amoxycillin in mouse CD_{50} tests, BRL 42715 showed particularly good synergistic activity against a wide range of β-lactamase producing organisms (*Table 6.16*). At the reported dosage level, sulbactam gave no useful protection to amoxycillin. Clavulanic acid and tazobactam gave good protection against infections produced by *Pr. vulgaris* and a strain of *E. coli* producing low levels of TEM-1, but were otherwise ineffective. BRL 42715 afforded good protection against all of the infections [93].

Early syntheses of the alkylidene and heterocyclylmethylene penems were lengthy and produced racemic compounds [94, 95]. For BRL 42715 (31), a synthetic route was established which (i) utilized the inexpensive and readily available chiral synthon, 6-APA (8) as starting material, (ii) retained the (5R) stereochemical integrity of 6-APA and as much of the original penam framework as possible, (iii) introduced the C-6 substituent at a late stage in the synthetic sequence, and (iv) was amenable to large scale preparation (*Scheme 6.17*) [96, 97].



Scheme 6.17. Synthesis of BRL 42715 [96, 97].

6-Aminopenicillanic acid (8) was converted to 6(*S*)-bromopenicillanic acid by trapping of the diazo-intermediate with hydrogen bromide. Esterification of the dicyclohexylamine salt (93) with *p*-methoxybenzyl bromide, followed by oxidation, afforded the sulphoxide (94) in 60% yield from 6-APA. Elaboration of this sulphoxide to the disulphide (96) was effected by the procedure established by Kamiya *et al.* [98]; the sulphenic acid (95), formed by heating the sulphoxide to reflux in toluene, was intercepted by reaction with 2-mercaptobenzothiazole to yield the disulphide (96). The latter was transformed by base-catalysed double bond isomerization to the conjugated ester disulphide (97) [95% yield from (94)]. Reductive formylation of disulphide (97) then provided the formylthio-derivative (98). Cyclization of the oxalimide (99), obtained by ozonolysis of

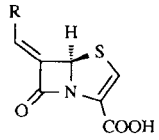


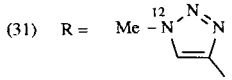
Scheme 6.17. (continued)

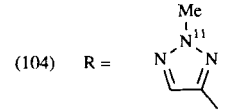
Reagents: (i) HBr, $NaNO_2$, MeOH–H_2O, 5°C, 1h; (ii) $HN(C_6H_{11})_2$, ether-light petroleum (2:1), 5°C, 16h; (iii) p-methoxybenzyl bromide, DMF, 25°C, 18h; (iv) m-chloroperbenzoic acid, MDC; (v) 2-mercaptobenzothiazole, toluene, reflux; (vi) Et_3N (cat.), toluene, 5°C, 2h; (vii) $MeCO_2CHO$ (10 equiv.), NaI (10 equiv.), DMAP, PPh_3, MeCN, −20°C to 5°C, 75 min; (viii) a) O_3, EtOAc, −78°C, 1h; b) aq, $NaHSO_3$; (ix) $P(OMe)_3$ (4 equiv), toluene, 95°C, 30 min.; (x) a) Ph_2NLi, THF, −78°C, immediately followed by 1-methyl-1,2,3-triazole-4-carbaldehyde, −78°C, 1 min.; b) Ac_2O, −78°C to 20°C over 10 min.; (xi) Zn, TMEDA · HCl, NH_2Cl (4 equiv), DMF, 20°C, 1 h; (xii) a) $AlCl_3$ (2.5 equiv), anisole, MDC, −40°C, 10 min.; b) Na_2HPO_4, −40°C to 20°C over 10 min. DMAP = *N,N*-dimethylaminopyridine; TMEDA = tetramethylethylenediamine.

the formylthioderivative (98), to the 6α-bromopenem ester (100) was effected by heating a toluene solution of (99) at 95°C for 30 minutes in the presence of trimethylphosphite [34% yield from (97)].

Sequential treatment of the bromopenem (100) with lithium diphenyl-







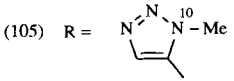
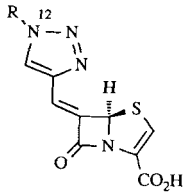


Table 6.17. SUMMARY OF BIOLOGICAL ACTIVITY OF ISOMERIC *N*-METHYL TRIAZOLYLMETHYLENE PENEM DERIVATIVES [100]

Compound	*I_{50} (μg/ml)*			*Amoxycillin MIC (μg/ml) in presence of 1 μg/ml of inhibitor*			*Relative potency in vivo*[b]
	E.cl. (Ia)[a]	*P.m. (II)*	*E.co. TEM-1 (III)*	*E.cl. (Ia)*	*P.m. (II)*	*E.co. TEM-1 (III)*	*E.co. TEM-1 (III)*
(31)	0.001	0.007	0.001	1	2	2	1.00
(104)	0.019	0.010	0.002	64	1	2	<0.05
(105)	0.055	3.500	0.017	128	>512	64	<0.05
Tazobactam	0.02	0.02	0.02	256	16	8	0.20
Amoxycillin alone				512	>512	>512	<0.05

[a]Enzyme classification based on Richmond-Sykes; [b]CD_{50} of amoxycillin in the presence of inhibitor relative to that of amoxycillin in the presence of BRL 42715 (31) in an *E. coli* (TEM-1) infection in mice; compounds were administered subcutaneously at 2 mg/kg with varying doses of amoxycillin at 1 and 5 hours post-infection (figures less than 1 indicative of inferior potency relative to BRL 42715). For abbreviations, see footnote to *Table 6.1*

amide, 1-methyl-1,2,3-triazole-4-carbaldehyde, and acetic anhydride gave a 4:1:1:1 diastereoisomeric mixture of acylated bromohydrins (101). Reductive elimination of this mixture using powdered zinc in N,N-dimethylformamide in the presence of *N,N,N′,N′*-tetramethylethylenediamine dihydrochloride and ammonium chloride afforded a separable 8:1 mixture of (*Z*)-



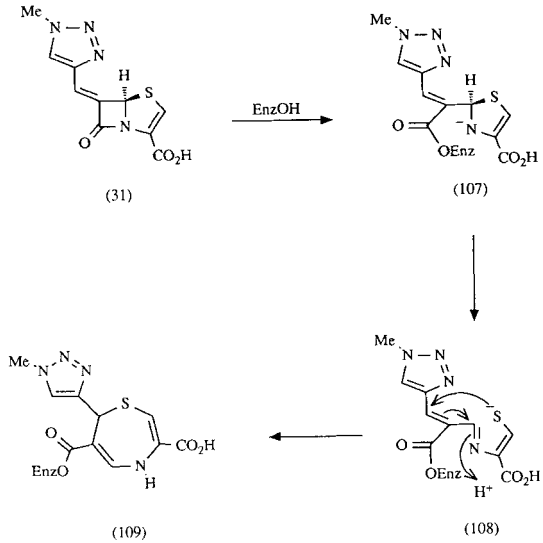
(106)

Table 6.18. BIOLOGICAL ACTIVITY OF *N*-12 SUBSTITUTED TRIAZOLYLMETHYLENE PENEMS [100]

Compound	*Amoxycillin MIC (μg/ml) in the presence of 1 μg/ml of inhibitor*					*Human serum binding (%)*	*Human kidney stability (%)*[b]	*Relative potency in vivo*[c]
	E.cl. (Ia)[a]	*P.m. (II)*	*E.co. TEM-1 (III)*	*K.p. (IV)*	*E.co. OXA-1 (V)*			
(31) BRL 42715	2	4	1	2	4	72	61	1.00
(106a) R=H	2	16	2	2	8	79	45	0.64
(106b) R=Et	2	4	2	2	4	72	72	0.28
(106c) R=n-Pr	32	2	2	4	8	81	67	0.04
(106d) R=cyclopropyl	4	2	4	4	8	80	82	0.17
(106e) R=CH_2CF_3	4	64	8	2	4	78	67	0.14
(106f) R=CH_2COONa	32	8	4	2	8	87	72	0.43
(106g) R=NMe_2	2	4	8	8	4	73	69	0.13
(106h) R=OMe	4	2	4	2	2	74	56	0.43
(106i) R=OH	4	16	8	8	16	72	90	<0.03
(106j) R=CH_2CH_2OH	4	4	2	2	4	56	69	1.00
(106k) R=$(CH_2)_3OH$	16	4	4	2	4	59	74	0.50
Tazobactam	256	16	8	16	>512	20	–	0.20
No inhibitor	512	>512	>512	256	>512	17	100	<0.03

[a] and [c] see footnotes in *Table 6.17*; [b]% remaining after incubation with human kidney at 37°C for 1 hour. For abbreviations, see footnote to *Table 6.1*.

and (*E*)-triazolylmethylene penem esters, (102) and (103) respectively, in 72% yield. Lewis acid-mediated deprotection of ester (102) provided the sodium salt of (5R)-(*Z*)-6-(1-methyl-1,2,3-triazol-4-ylmethylene)penem-3-carboxylic acid (BRL 42715) (31) in 75% yield.



Scheme 6.18. Proposed mechanism of action of BRL 42715 [101].

This synthetic route preceeded *via* the novel and versatile intermediate, the 6(*S*)-bromopenem ester (100) and permitted not only the preparation of large quantities of BRL 42715, but also the preparation of a series of chiral 6-heterocyclylmethylene penem derivatives [99, 100].

Tables 6.17 and 6.18 show the comparative inhibitory activity of a series of triazolylmethylene penems prepared by the above synthetic route. *Table 6.17* shows the effect on biological activity of altering the position of the methyl group on the triazole ring. The *N*-12-methyl derivative, as in BRL 42715, was the most active of the triazole derivatives. *Table 6.18* demonstrates the effect of varying the alkyl substituent at the N-12 position of the triazole ring. There was little difference in the synergistic activity with amoxycillin shown by these compounds, all of them proving to be better broad spectrum synergists than clavulanic acid, sulbactam and tazobactam [100]. This increased potency was most noticeable against organisms producing the class I or OXA-1 enzyme. Most were reasonably stable to human kidney homogenate and none showed excessively high binding to human serum. However, only the 6-[1-(2-hydroxyethyl)-1,2,3-triazol-4-ylmethylene]penem (106j) showed *in vivo* activity equal to that of BRL 42715.

The mechanism of action of BRL 42715 has been investigated by Broom

et al [101]. Studies involving the base-catalysed methanolysis of BRL 42715, as well as its interaction with the K1 β-lactamase from *K. pneumoniae* 1082E, suggest initial attack at the β-lactam carbonyl by the active-site serine hydroxyl, followed by C(5)-S bond fission to provide the ene-thiolate (108), which undergoes an intramolecular Michael reaction to yield the dihydrothiazepine acyl-enzyme complex (109) (*Scheme 6.18*). This rearrangement product contains the same vinylogous urethane subunit believed to confer stability to the acyl-enzyme formed after the interaction of β-lactamases with other suicide inhibitors such as clavulanic acid and sulbactam. It was therefore concluded that the formation of the dihydrothiazepine acyl-enzyme complex (109) is responsible for the irreversible inactivation of bacterial β-lactamases by BRL 42715.

CLINICAL IMPLICATIONS AND COMMERCIAL ASPECTS

For commercial success, any β-lactamase inhibitor must have the right balance of *in vivo* activity, stability, pharmacokinetic profile, tolerance, safety and cost-efficiency, as well as demonstrating *in vitro* activity against isolated enzymes and intact bacteria. To date, only clavulanic acid and sulbactam have fulfilled these stringent criteria.

Clavulanic acid is manufactured by SmithKline Beecham and is sold as oral and parenteral products in combination with amoxycillin under the trade name of Augmentin, and as an injectable product in combination with ticarcillin under the trade name of Timentin. Augmentin is prescribed for infections of the respiratory tract such as bronchitis, and ear, nose and throat infections such as otitis media and sinusitis. It is also indicated for infections of the urinary tract, such as gonorrhoea, and skin and soft tissue infections, which include cellulitis, impetigo and abscesses. Timentin meanwhile is used for the treatment of severe infections in hospitalised patients with impaired or suppressed host defences.

By the end of 1992, Augmentin was the second best selling antibiotic worldwide and combined sales of oral and parenteral products exceeded £500 million. The total audited sales of clavulanate-based products for 1992 approached £600 million [102].

Sulbactam is marketed by Pfizer, in combination with ampicillin under the name of Unasyn and in combination with cefoperazone under the name of Sulperazon, both as injectable products. The oral version of sulbactam is the mutual pro-drug of sulbactam and ampicillin (sultamicillin) and is sold by Pfizer under the brand name of Unasyn Oral. Uses of Unasyn Oral are similar to those of Augmentin and include infections of the upper and lower

respiratory tract, urinary tract and skin and soft tissue. Injectable Unasyn is indicated for the treatment of severe infections such as pyelonephritis and septicaemia and for intra-abdominal infections, as well as in surgical prophylaxis. By the end of 1992, Unasyn was the 12th best selling antibiotic worldwide and combined parenteral and oral sales exceeded £145 million. Together with Sulperazon, sales of sulbactam-based products exceeded £210 million [102].

Tazobactam has recently been launched by Lederle in the U.K. as a combination with piperacillin under the trade name of Tazocin. This product is indicated for urinary tract infections, lower respiratory tract infections and intra-abdominal, biliary and cutaneous infections.

The 6-heterocyclylmethylene penems, represented by BRL 42715, are still the most potent class of β-lactamase inhibitors known to date. Their potential is still under active investigation.

CONCLUSIONS

The discovery of clavulanic acid in 1976 heralded a new era in antibacterial chemotherapy. The enormous success of Augmentin (amoxycillin in combination with clavulanic acid) for the treatment of infections caused by β-lactamase producing bacteria has established that β-lactamase inhibitors have an important role to play in solving the problem of bacterial resistance. The widespread interest and enthusiasm created by the discovery of clavulanic acid has culminated in the development of two additional β-lactamase inhibitors, namely, sulbactam and tazobactam.

More recently, the identification of the 6-heterocyclylmethylene penem class of inhibitors has confirmed that medicinal chemists and microbiologists are more than prepared to meet the perpetual challenge of emerging bacterial resistance.

ACKNOWLEDGEMENTS

The authors are grateful for the helpful comments made by many colleagues, in particular Drs. R.J. Ife, F.D. King, B.W. Metcalf, P.J. O'Hanlon, M.J. Pearson and T.C. Smale. We also wish to thank Mrs. T. Wilkins and Mr. C. T. Coulton for their help in preparing this manuscript.

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Progress in Medicinal Chemistry – Vol. 31, edited by G.P. Ellis and D.K. Luscombe

7 Antimicrobial Activity and Action of Silver

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INTRODUCTION

Silver and its compounds have long been used, in one form or another, as antimicrobial agents. The silver compound of major therapeutic interest at the present time is silver sulphadiazine. Many in health care, however, will

know that other silver compounds are still in use. It is worth stating that the treatment of ophthalmia neonatorum was revolutionized by the instillation of silver derivatives into the eyes of new-born sufferers.

Silver compounds also have non-medical uses, several of which will be considered. In this short review, we shall trace their pedigree, describe their uses and discuss in depth their mechanisms of action and of bacterial resistance to them.

HISTORICAL ASPECTS

A very early, perhaps the first, report of the use of silver appeared in pre-dynastic Egypt around 3500 BC, where it was used for currency. Medicinally, it has been known since 1000 BC, and possibly before, that water kept in silver or copper vessels, exposed to light or filtered, could be rendered potable [1]. Certainly Alexander the Great (335 BC) was advised by Aristotle to store water in silver vessels and to boil it before use when he transported it on his many campaigns. It must be inferred that the antimicrobial activity of copper and silver was being exploited, albeit empirically, in those very early days and must have been based on shrewd observation.

Following the empirical use of silver in classical antiquity, Ravelin in 1869 [2] was the first to report on the very low concentrations at which silver and other metal derivatives could exert their antimicrobial effect. The word 'oligodynamic', literally 'active with few' (molecules, ions) was coined by von Naegeli [3] in 1893 to denote this activity. von Naegeli found that a concentration of silver ions, derived from metallic silver, of 0.0000001%, equivalent to 9.2×10^{-9} M, would kill the common fresh-water *Spirogyra*. At a concentration of 0.00006%, equivalent to 5.5×10^{-6} M, the germination of *Aspergillus niger* spores was prevented.

From the point of view of therapeutic use, it is interesting to note that, in 1881, Credé [4] had published a monograph advocating the use of silver in the prevention of eye infections in neonates. The oligodynamic action of silver can be demonstrated by pouring nutrient agar, seeded with a suitable test organism, over a strip of silver contained in a Petri dish. After incubation, a zone of inhibition will be found around the strip. Gibbard [5] investigated in some detail those factors that affected the antimicrobial activity of metallic silver. Interestingly, if silver is cleaned mechanically with abrasive-coated cloth or paper, it becomes inactive. Similarly, if molten silver is allowed to cool in an atmosphere of hydrogen, no activity is

apparent, whereas cooling in air yields an active metal. Treatment of silver with nitric acid enhances its activity. The general conclusion to be reached from this set of experiments was that pure silver is devoid of activity but that tarnished and/or surface-oxidised silver was active. Acél [6] had suggested that the oligodynamic action of silver was due to the liberation of Ag^+ ions. Gibbard [5] also found that colloidal silver, silver oxide and silver nitrate were always active. He further demonstrated that the general antimicrobial properties of silver and its derivatives were reduced in the presence of protein. Thus by 1937 the factors governing von Naegeli's oligodynamic action of silver had been elucidated and the inhibitory action of protein observed. Additional papers on the topic from Ravelin's published work in 1869 [2] to Gibbard's in 1937 [5] have been reviewed by Romans [7].

SILVER COMPOUNDS

The first record of the medicinal but not antimicrobial use can be found in writings around 750 AD. An early scientific paper by Credé has already been alluded to [4]; it involved the use of silver in the treatment of inflammation of the eyes (ophthalmia neonatorum) in new-born infants. In a further paper [8], silver was advocated as an internal antiseptic. During the following years, silver nitrate, citrate, lactate and proteinate have appeared in pharmacopoeias and formularies around the world. A silver nitrate lotion appeared in older editions of the British National Formulary. In the latest issues, however (no. 26, 1993 onwards: [9]) only silver sulphadiazine is to be found. The USP XXII [10] lists silver sulphadiazine (used as a cream) and silver nitrate (used as an ophthalmic solution).

ARGYRIA

Argyria, also called argyrism or argyrosis, which occurs as a result of prolonged silver therapy, was first reported in 1647 [11], and was reviewed in depth by Hill and Pillsbury [11] in 1939. It presents as an irreversible grey to blue-black colouring of the skin and mucous membranes, for example, the conjunctiva, and also of internal tissue. With the decline in use of many silver preparations in therapeutics, the condition is less likely to be encountered today. However, a recent case has been reported in a 59-year old patient undergoing silver sulphadiazine therapy for recurrent venous leg ulcers [12].

PROPERTIES AND USES OF SILVER COMPOUNDS

SILVER AND SILVER SALTS

The following silver and silver compounds are listed in Martindale, The Extra Pharmacopoeia [13]: Silver metal, silver acetate, silver nitrate, silver protein and silver sulphadiazine. All possess antimicrobial activity. Silver acetate and especially silver nitrate possess disinfectant properties; silver nitrate has been employed as a 1% w/v solution for the prophylaxis of ophthalmia neonatorum, but its irritating properties and lack of activity against chlamydia tend to militate against its use. Silver nitrate in stick form has been used to destroy warts, as a lotion for suppurating lesions and as a compress to reduce infection when applied to severe burns. Although listed until recently in the British National Formulary, BNF, silver nitrate has been deleted from the latest issues of the BNF [9]. Silver protein solutions are antimicrobial as a consequence of the presence of low concentrations of ionized silver and have been employed as eye-drops. It is less effective than silver nitrate [14, 15]. Other silver salts include the lactate, picrate and sulphate, which are no longer used medicinally. In addition to being used in human medicine, silver salts have been employed in the veterinary field as general veterinary antiseptics and for the cauterization of small wounds [16, 17].

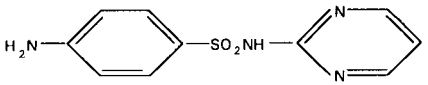
The antimicrobial properties of silver and its salts have been discussed in various books [14, 16–18] and particular reference must be made to Grier's review in 1983 [15]. Ag^+ usually used in the form of silver nitrate, is bacteriostatic or bactericidal [19–29], antifungal [30–32], protozoicidal [33] and lethal to herpes simplex virus [34]. However, bacterial spores [19], cysts of *Entamoeba histolytica* [19] and mycobacteria [35] are not killed by Ag^+. Brown and Anderson [20] observed a non-linear order of death in *Pseudomonas aeruginosa* exposed to Ag^+, whereas Ricketts *et al.* [22] reported a rapid bactericidal action of Ag^+ in water, but not in broth, at concentrations of silver nitrate of 0.5 and 1 μg/ml (2.9×10^{-6} and 5.8×10^{-6} M, respectively) with inactivation at concentrations above 1 μg/ml being too fast for measurement.

Several factors influence the antimicrobial activity of silver salts [19]. Silver has a marked tendency to adsorb to surfaces and bactericidal activity is reduced in the presence of phosphates, chlorides, sulphides and hard water. Activity is increased as the temperature is raised ($Q_{10} = 1.6$, that is, a 1.6-fold increase in activity per 10°C rise in temperature) and is pH-dependent, increasing with increasing pH [19, 20]. Sodium thioglycollate has been recommended as a suitable neutralizing agent for use in

bactericidal testing [19, 36] although other SH compounds also fulfil this role [37].

SILVER SULPHADIAZINE

Water-soluble compounds such as sodium sulphadiazine (NaSD) are known to be absorbed rapidly from wounds and excreted in the urine. Attempts were therefore made in the 1960s to retard the absorption of sulphadiazine (SD) (1), as a result of which silver sulphadiazine (AgSD) among other compounds was synthesized. SD is a weak acid and reacts with silver nitrate to produce a water-insoluble silver salt, AgSD [38], to which a particular polymeric chemical structure has been assigned [39]. X-ray crystallography revealed that this polymer was made up of six silver atoms bonded by linkage to nitrogens in the pyrimidine ring to six SD molecules. Of the compounds prepared and tested, only AgSD was effective topically in an ointment base against *Ps. aeruginosa* in an animal burn model [40].



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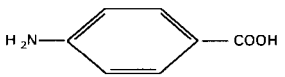


(2)

AgSD differs from other silver salts in its physical properties: it does not react rapidly with chloride or thiol groups or with protein and thus its antibacterial activity is not reduced. It does not become dark on standing or in contact with body tissues and unlike other sulphonamides its activity is not eliminated in the presence of *p*-aminobenzoic acid (PABA) (2) [40].

The antimicrobial activity of AgSD has been described by several authors [26, 41–56]. It inhibited all bacterial strains tested by concentrations easily achieved topically; furthermore, it was active against strains resistant to NaSD and to strains possessing multiple antibiotic resistance [43, 44]. Combinations of dibromopropamidine isethionate (DBPI) and silver nitrate or AgSD are antagonistic or synergistic, depending upon the proportions of DBPI to silver compound [57, 58]. AgSD promotes the rapid healing of

wounds [56] and is useful in the treatment of severe burns [59] and of chronic pressure ulcers [60].

CLINICAL AND OTHER USES OF SILVER COMPOUNDS

The clinical usefulness of silver compounds was briefly alluded to above. The reintroduction in 1965 of silver nitrate for topical antimicrobial prophylaxis of burns [61] was an important development. This finding was confirmed and extended in a series of papers presented by Lowbury and his colleagues [62–68]. They demonstrated the effectiveness of silver nitrate compresses against *Ps. aeruginosa*, although other organisms such as *Klebsiella* spp. were resistant. In controlled trials in Birmingham, they further found that AgSD had comparable effects to silver nitrate compresses in severely burned patients and that AgSD had a much greater inhibitory effect against *Klebsiella* spp., although less against *Staphylococcus aureus*, than silver nitrate. Subsequently, however, plasmid-mediated sulphonamide resistance emerged in enterobacteria and the use of all sulphonamides, including AgSD, was suspended. AgSD was replaced by a prophylactic cream containing silver nitrate and chlorhexidine; resistance to these two components of the cream did not occur and interestingly, the levels of sulphonamide-resistant enterobacteria fell to levels found before the introduction of AgSD into the unit. These findings are comprehensively discussed by Lowbury [69] whilst mechanisms of resistance to silver compounds, including AgSD, are considered later in the present paper. AgSD has been listed [70] as an alternative to hexachlorophane for reducing staphylococcal colonization and infection of neonates. A silver-releasing inorganic polymer for preventing nosocomial bacteriuria has been recommended [71] as a routine, prophylactic measure for improving the health of short-term catheterized patients.

Inorganic silver salts/metallic silver also find use in non-clinical areas. The use of silver as a drinking-water disinfectant is much more popular in Europe than in the United States [72]; the same is true for swimming pool disinfection [73, 74], although there has been a resurgence of interest in the latter aspect. Electrically generated Cu^{2+} and Ag^{+} ions reduce viable numbers of *E. coli, Staph. aureus* and *Enterococcus faecalis* especially when chlorine is present [75]. The chlorine concentration can be reduced although still able to meet guidelines for swimming pool disinfectants [75], and the combined system has been evaluated and found to be satisfactory for indoor and outdoor pools [76]. However, because of factors such as trihalomethane formation and possible bacterial regrowth, alternatives to chlorine have

been sought. Electrically generated Cu^{2+} and Ag^{+} ions with low levels of iodine have proved to be satisfactory [77].

Activated carbon, silver-impregnated filters have also been described [78, 79] and have been shown to be effective in preventing growth of, or killing, *Ps. fluorescens* and *Ps. aeruginosa* in water supplies.

MECHANISMS OF ANTIMICROBIAL ACTION

Early empirical use and scientific studies made during the last decade of the nineteenth century had shown that silver was an extremely active biocide at silver ion (Ag^{+}) concentrations of approximately 10^{-9} to 10^{-6} M. Evidence that this was due to the silver ion was presented in 1937 [80]. From this, the question arises of how or by what mechanism does the silver ion kill micro-organisms. As with many antimicrobial drugs, the term 'general protoplasmic poison' was applied to the activity of silver. Equally, with increasing knowledge of cell metabolism and cell physiology and structure, and with increasingly elaborate techniques for probing cellular mechanisms, this conclusion was found to be a gross over-simplification. Whilst it is true that the protoplasm is indeed 'poisoned', there is much to be learned as to how this may occur. The next development was the realization that silver reacted strongly with thiol (sulphydryl, SH) groups in the bacterial cell, whether they be in structural or functional (enzymic) proteins. Finally, it was demonstrated that silver produces structural changes in bacterial cells and interacts with nucleic acids. All of these aspects are considered together with the special case of AgSD.

EFFECTS ON ENZYMES

Yudkin [80] examined the action of silver nitrate on the metabolic activity of *Bacterium (Escherichia) coli* by following the reduction of methylene blue and its inhibition using the then widely-employed Thunberg technique and single substrates. He observed that silver (10 μm) inhibited glucose, succinate and lactate oxidation (dehydrogenation) and that, in the case of glucose, the oxidation was initially stimulated before inhibition. Today, this would indicate the possibility of an uncoupling effect. Later, Bragg and Rainnie [81] confirmed these findings but used an oxygen-uptake monitor rather than methylene blue reduction to measure substrate oxidation. These workers also demonstrated that reduced glutathione prevented the inhibitory action of silver, suggesting that sulphydryl enzymes were a site of silver's activity. They concluded that the most sensitive site for silver action

lay between cytochrome b and a_3, with a second, less sensitive, site between NADH and succinic dehydrogenase. They further suggested that silver had a mode of action similar to that of mercury [82]. A silver-sensitive site lying between NADP and glutamic dehydrogenase was described in *Trepanosoma cruzi* [83]; this inhibition also was reversed by reduced glutathione.

Silver ion has been found [84] to inhibit phosphate uptake in *E. coli* and to bring about efflux of accumulated phosphate.

Furthermore, if the cells had been grown in the presence of mannitol, succinate, glutathione and proline and had accumulated these substrates, Ag^+ promoted their efflux. All these effects were reversed or prevented by thiols. In the presence of the uncoupling agent, carbonyl cyanide-*m*-chlorophenylhydrazone (CCCP) further inorganic phosphate (Pi) uptake was inhibited and Pi efflux no longer occurred. In contrast, the uncouplers carbonyl-cyanide-*p*-trifluoromethoxyphenylhydrazone, tetrachlorosalicylanilide (TCS) and 2,4-dinitrophenol (DNP), while blocking Pi uptake, did not prevent Pi efflux.

INTERACTION WITH SH GROUPS

The information presented above provides evidence that a prime molecular target for the silver ion resides in cellular SH groups. Such interaction, depicted in *Figure 7.1*, as well as reversal by SH groups, would then be responsible for causing bacterial inhibition.

It is now pertinent to consider more deeply the interaction of Ag^+ with thiols. It has long been realized that heavy metal ions interact with cell membrane-associated enzymes [15, 36, 37, 85–105]; such metals include mercury, silver, copper and lead. The thiol group derived from cysteine residues is essential for the activity of many enzymes [102]; many metals and biocides interact with this essential group in some way. These include the following interactions with RSH [99, 102]:
(a) reversible oxidation to produce disulphides (3), for example, with hydrogen peroxide and iodine; (b) irreversible oxidation to produce

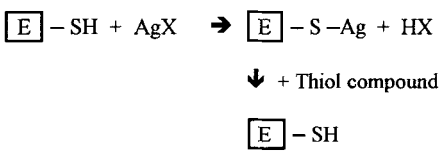
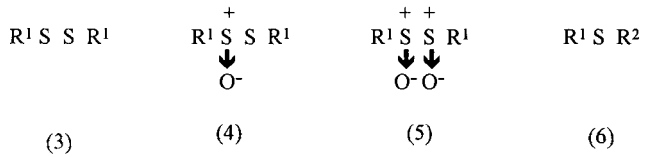


Figure 7.1. Interaction of silver with SH groups of an enzyme (E) and its reversal by thiol compounds.

disulphides, followed by sulphoxides (4) and disulphoxides (5), for example, with halogens; (c) alkylation (6), for example, by glutaraldehyde (pentanedial) (7) and formaldehyde (8).

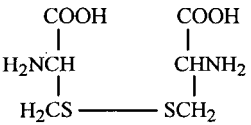


(3) (4) (5) (6)

(7) (8)

In these interactions, R^1 is protein-bound and R^2 represents an alkyl group. In addition, mercaptide (10) formation (in which X represents the inhibitor), as depicted in *Figure 7.2* occurs with various metals, notably mercury and silver, and with cysteine (9).

(9) (10)



(11)

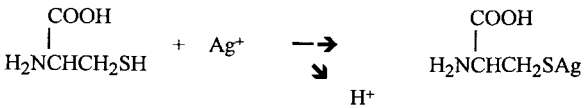
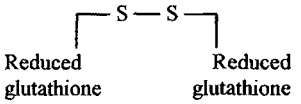
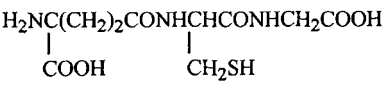


Figure 7.2. Interaction of cysteine with Ag^+

Cystine (11) also plays a special role in protein structure, because its disulphide group serves as a covalent cross-link between two polypeptide chains [99]. Mercaptoethanol and other disulphide bond-breaking agents cleave such cross-links. Disulphide bonds are much less active than sulphydryl (thiol) groups and function as stable elements of protein structure [95]. Most SS and SH groups in nature are contributed by L-cystine (CySSCy) and L-cysteine (CySH) in various combined forms. It has been demonstrated that hydrogen bonding may also be important, because the rate of reaction of Ag^+ with oxidized glutathione (GSSG) (12) and glutathione (GSH) (13) is more rapid than the rate of reaction with CySH (or CySSCy). However, in the presence of urea (a hydrogen bond-breaking agent), the former rate falls to that in the latter [95].



(12)



(13)

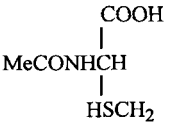
The specificity of Hg^{2+} and Ag^+ for SH groups is, however, only relative; when they are added to a protein solution, the SH binding sites are first saturated, but any excess metal ions will immediately begin associating with other groups [91]. The inhibitory effects of a number of metal ions has been related directly to the logarithm of the solubility constant of metal sulphides; Ag^+ was considerably more effective than Hg^{2+}, an observation which has been ascribed to the greater ability of the former to react with masked SH groups [86]. Metal ions, for example, Hg^{2+}, Ag^+ and Cu^{2+}, which form acid-stable sulphides with hydrogen sulphide, H_2S, also form stable compounds of low solubility with thiols [95].

It is now pertinent to consider the specific role of thiol groups in the effect of Ag^+ on micro-organisms. The effect of Ag^+ can be overcome by compounds containing SH groups, for example, thioglycollic acid (14), cysteine (9), reduced glutathione (13), *N*-acetyl-L-cysteine (15) and the disulphide bond-containing L-cystine dimethyl ester (16) but not by other compounds containing S-S, S, or SO_3H groups, for example, cystine (11), cystamine (17), cystathione (18), cysteic acid (19), methionine (20) and taurine (21) [37].

With the exception of L-cystine dimethyl ester (16), these results show the importance of the SH group in the interaction of silver with amino acids or

$HSCH_2COOH$

(14)



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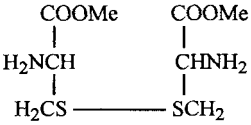
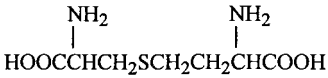


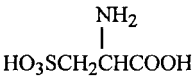
(16)

$H_2N(CH_2)_2S{-}S(CH_2)_2NH_2$

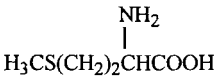
(17)



(18)



(19)



(20)

$NH_2(CH_2)_2SO_3H$

(21)

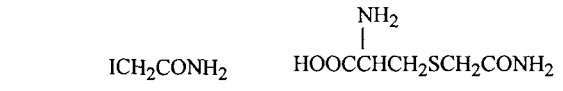
similar compounds. Furthermore, iodoacetamide (22) combines with CySH to produce CyS-acetamide (23), which no longer possesses a thiol group and which does not reverse the antibacterial action of silver [37]. Some of the compounds listed are worthy of further comment: thus, glutathione (GSH) (13) is a tripeptide that contains cysteine, glutamic acid and glycine residues; neither glutamic acid nor glycine reverses the antibacterial activity of silver [37]. Taurine is formed from cysteine by oxidation and decarboxylation.

Silver ion causes the release of K^+ ions from erythrocytes [106] and the microbial plasma or cytoplasmic membrane, with which is associated many important enzymes, is an important target site for the metal [84, 95, 96, 107,

109]. Those metals (Hg^{2+}, Ag^{+}, Cu^{2+}) that react with SH groups penetrate the membrane rapidly, interfering with membrane functions and also interacting with internal SH groups so that the function of many enzymes is inhibited [107]. It is, however, debatable as to whether Ag^{+} acts solely as an uncoupling agent, as an inhibitor of the respiratory chain or as a thiol reagent [84].

CYTOLOGICAL CHANGES

In addition to its effect on enzymes, Ag^{+} has been found to produce changes in micro-organisms which may or may not be related. Thus, silver nitrate caused marked growth inhibition of *Cryptococcus albicans*, and was



(22) (23)

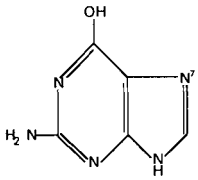
deposited in the vacuole and cell wall as granules [32]. X-ray microanalysis revealed that the low level of chloride was present in the wall. These findings imply [32] that the uptake of Cl^{-} into the cytoplasm was associated with silver toxicity but that silver was bound to the wall in some other way.

Silver nitrate inhibits cell division and damages the cell envelope and contents of *Ps. aeruginosa* [110]; sensitive cells increased in size and the cytoplasmic contents, cell membrane and outer cell layers all presented abnormalities. A strain of *Ps. aeruginosa* which was resistant to AgSD was much less affected by Ag^{+} in the form of silver nitrate [111]. Spheroplasts of the resistant strain were less susceptible than the sensitive strain to lysis by silver nitrate, which implies that the cytoplasmic membrane is a possible site of silver resistance.

INTERACTION WITH NUCLEIC ACIDS

Yet another effect of the action of Ag^{+} has been described, namely, its interaction with nucleic acids [112]. The Ag^{+} ion interacts preferentially with the bases found in DNA rather than with the phosphate group [113–118]. The reaction between Ag^{+} and a GC (guanine–cytosine) base pair proceeds via two steps, whereas that between Ag^{+} and an AT (adenine-thymine) base pair requires only one step [116]. Ag^{+} appears to be attached to the N^{7} atom

of guanine (24), and there is a strong suppression of the DNA-Ag^+ complex involving GC in calf thymus DNA highly methylated at this guanine site [115].



(24)

The photochemical response of bacterial DNA and of synthetic polynucleotides to UV, and of the influence thereon of Hg^{2+} and Ag^+ have been examined [119, 120]. The photochemistry of DNA is altered greatly when these metals are bound, but in different ways. Hg^{2+} complexed with bacterial DNA greatly reduces the rate of thymine dimerization, whereas Ag^+ binding to DNA greatly enhances dimerization. Consequently, biological inactivation is increased in the presence of Ag^+ but reduced with Hg^{2+}.

If this enhancement of dimerization in UV-exposed DNA is transposed to the more normal situation that applies when bacteria are treated with a silver salt such as $AgNO_3$, then it is tempting to speculate that interaction of DNA with Ag^+ is a primary target site. It would, however, be premature to propose that such an interaction is necessarily responsible for bacterial inactivation, although it must clearly play some role.

SILVER SULPHADIAZINE

AgSD differs markedly from silver nitrate in that the former is essentially a combination of two antibacterial agents, Ag^+ and SD. The question may then be posed as to whether the antibacterial effect results predominantly from one of the components or by a synergistic interaction. AgSD has a broad spectrum of activity [26, 41–56] and, unlike silver nitrate, produces surface and membrane blebs (surface protuberances) in susceptible but not resistant bacteria [44]. Binding of AgSD to cell components, especially DNA, has been demonstrated [42, 46]. When S^{35}-labelled SD was incorporated into medium inoculated with *Ps. aeruginosa*, no labelled SD was found in either the bacterial cells or the DNA fraction [40, 50]; in contrast, when ^{110}Ag-labelled SD was employed, the labelled silver was found only in the DNA fraction [50]. Other properties of AgSD are also of

interest. It does not appear to be mutagenic [121], its activity is not reduced by PABA (2), unlike other sulphonamides [105], and thus it does not possess a mode of action typical of the sulphonamide group of drugs. However, the SD portion of AgSD contributes markedly to the activity of AgSD cream [57] and sulphonamide-resistant clinical isolates are also resistant to AgSD [57, 68]. Fox [40] has considered the effect of AgSD on DNA in the context of a polymeric structure of AgSD, which is composed of six silver atoms bonding to six SD molecules by linkage of the metal atoms to the nitrogens of the pyrimidine ring. Bacterial inhibition would presumably be achieved when silver binds to sufficient base pairs in the DNA helix.

Despite AgSD having now been in use for more than 20 years, it is clear that its precise mechanism of action has yet to be elucidated.

BACTERIAL RESISTANCE TO SILVER

In general terms, bacterial resistance to antibacterial agents can be considered as being either intrinsic (innate, a natural property) or acquired, for example, by mutation or by the acquisition of a plasmid or transposon (jumping gene) [105]. Resistance to mercury [103, 122–125] and to other cations and anions [103, 122, 123, 125] is well characterized. The mechanisms involved in resistance to some metals, including silver, are summarized in *Table 7.1*.

Resistance to silver also occurs but the mechanisms for this reduced

Table 7.1. MECHANISMS OF BACTERIAL RESISTANCE TO SILVER AND OTHER METALS

Metal	*Organism*	*Plasmid-encoded*	*Mechanism*
Silver	*E. coli, Salmonella typhimurium*	Yes	Decreased uptake
	Pseudomonad	No	Silver detoxified by reduction to metallic form
Mercury (inorganic)	Various	Yes	Mercuric reductase
Mercury (organic)	*E. coli, Ps. aeruginosa Staph. aureus*	Yes	Mercuric hydrolase and reductase
Cadmium	*Staph. aureus*	Yes	cadA system: efflux by ATPase pump

sensitivity are not altogether clear (*Table 7.1*) In the case of AgSD, however, resistance could be due to the silver component, the sulphonamide component or both components. Mycobacteria [35] and bacterial spores [19] are intrinsically resistant to Ag^+ although the mechanisms involved are unclear; impermeability to Ag^+ may be involved. Acquired resistance to Ag^+ in *E. coli* by chromosomal mutation is associated with a decreased silver binding by a major outer membrane protein [126]. Plasmid-encoded resistance to silver has been described by several authors [103, 124, 125, 127–134] in Enterobacteriaceae, *Pseudomonas* spp and *Citrobacter* spp. However, the mechanisms involved remain poorly understood. Attempts to transfer silver resistance from silver-resistant (Ag^R) strains of *E. coli* and *Klebsiella* to silver-sensitive (Ag^S) strains of *E. coli* were unsuccessful [127]. The difficulty of transferring silver resistance from Ag^R to Ag^S has been pointed out elsewhere, although it can be achieved [128] to produce a very wide ratio of minimum inhibitory concentrations (MICs) of >100 : 1 for Ag^R : Ag^S cells. The basis of mercury (Hg^{2+}) resistance encoded by plasmids is well understood: Hg^{2+} is converted by the enzyme mercuric reductase to mercury (Hg^o) which then vaporizes [102, 122, 123, 125]. Likewise with phenylmercuric compounds, Hg^{2+} is released (the enzyme involved is a hydrolase) and is then converted as before to Hg^o which again vaporizes. Plasmid-encoded silver reduction, analogous to Hg^{2+} reduction, has not been demonstrated. Plasmid-mediated efflux pumps have been described for cadmium and arsenate and indeed for acridines and quaternary ammonium compounds (105, 132, 134, 135], so that the intracellular concentrations of these compounds are reduced. No efflux pump has as yet been detected in silver-resistant bacteria [134].

One clue about a possible mechanism of silver resistance has been obtained from studies involving silver accumulation, although this need not necessarily apply only to plasmid-mediated resistance. Silver accumulation in some bacteria is relatively high [136, 137] and is probably energy-dependent [134]. *Thiobacillus ferro-oxidans* and *T. thio-oxidans* accumulated high levels of Ag^+ [137], and the membrane was covered with electron-dense silver sulphide particles. Contrary, therefore, to the proposal that only Ag^S bacteria accumulate silver [122], it is clear that Ag^R cells can also accumulate high concentrations which might be beneficial biotechnologically [134]. However, sensitive (but not resistant) bacteria appear to compete successfully with silver salts for the acquisition of silver ions [125]. It was pointed out above that plasmid-encoded Ag^+ reduction, analogous to Hg^{2+} reduction and vaporization, had not been described. However, Belly and Kydd (138] isolated silver-resistant bacteria, yeast and fungi from photographic sludges and other sources and investigated one organism (a

pseudomonad) in detail. In this strain, silver accumulated in cells predominantly as Ag^{o}, Ag_2O or Ag^{+} but not as Ag_2S or AgCl, from which the authors postulated that silver had been detoxified by its reduction to the metallic form and by its complexation with cellular components. Cell-free extracts of this organism were unable to reduce Ag^{+} to Ag^{o} and although this pseudomonad was mercury-resistant, there was no evidence that a mercury reductase could reduce silver, as had earlier been reported in a plasmid-containing mercury-resistant *E. coli* [139]. Clearly, much remains to be discovered about the mechanism(s) of silver resistance. Silver resistance does not appear to be linked to chlorhexidine resistance [140]. Resistance to sulphonamides, including AgSD, has been described [68, 69], is transferable [66], and has been reported in many countries. The mechanism of this resistance is unclear, but could be associated with sulphonamide-insensitive enzymes and/or to reduced uptake of Ag^{+}.

CONCLUDING REMARKS

Silver, one of the native metals and second only to gold in its stability amongst the metals of antiquity, has provided several therapeutic agents which have been employed since the beginning of recorded history. These agents range from the metal itself, its salts and complexes with proteins and other macromolecules to the latest, AgSD.

Studies of the antimicrobial and therapeutic properties of silver and its derivatives have yielded an extensive scientific literature, ranging from clinically- to biochemically-based papers. The latter area includes studies in enzymatic and other macromolecular interactions to silver resistance encompassing plasmid-mediated patterns. The reduction to one silver compound (AgSD) in the current British National Formulary and to two compounds (AgSD and silver nitrate ophthalmic solution) in USP XXII may not be a true reflection of the use of silver in health care today. Silver compounds also find use in non-clinical situations, for example, as water disinfectants and, not considered in this review, as silver staining techniques for microscopy.

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Progress in Medicinal Chemistry – Vol. 31, edited by G.P. Ellis and D.K. Luscombe

8 Inhibition of the Pharmacological Effects of Endothelin

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INTRODUCTION

Endothelin is one of the most potent vasoconstrictors known and its effects on the vasculature are characterized by an unusually long duration of action. Endothelin is also a potent cellular mitogen and a constrictor of non-vascular smooth muscle. With such profound biological actions, it is of little surprise that a vast amount of effort has been directed towards establishing the physiological or pathological role of endothelin and towards inhibiting its actions. The pharmacological effects of endothelin can be prevented either by inhibiting the formation of endothelin or by blocking the action of endothelin at its receptors. The two approaches are discussed in later sections.

Endothelin was first identified by Yanagisawa *et al.* [1] as a 21 amino acid peptide which was released from porcine aortic endothelial cells. This mature form of endothelin, which contains two disulphide bridges between residues Cys^{1}-Cys^{15} and Cys^{3}-Cys^{11}, is derived from a 39 amino acid precursor, 'big endothelin', by means of a putative endothelin converting enzyme *(ECE) which cleaves the Trp^{21}-Val^{22} bond (*Figure 8.1*). Big endothelin itself is derived from a 203 amino acid precursor by means of dibasic pairspecific endopeptidases.

Subsequently it was found that the human genome encodes for three endothelin isoforms, one of which, endothelin-1 (ET-1), is identical to the peptide which was isolated from porcine aortic endothelial cells [2]. Endothelin-2 (ET-2) is [Trp^{6},Leu^{7}]ET-1 and endothelin-3 (ET-3) is [Thr^{2},Phe^{4},Thr^{5},Tyr^{6},Lys^{7},Tyr^{14}]ET-1 (*Figure 8.2*). Human big ET-1 is a 38 amino acid peptide and differs from porcine big ET-1 in that it lacks Ser^{37} and has Val^{28} instead of Ile^{28} (*Figure 8.1*). The endothelins also bear remarkable similarity, both in structure (*Figure 8.2*) and biological activity,

*The following abbreviations are used in this chapter: ACE, angiotensin converting enzyme; CNS, central nervous system; DNA, deoxyribonucleic acid; ECE, endothelin converting enzyme; ET-1, endothelin-1; ET-2, endothelin-2; ET-3, endothelin-3; GFR, glomerular filtration rate; GI, gastrointestinal; PTCA, percutaneous transluminal coronary angioplasty; RCM, radiocontrast media; RIA, radio-immuno assay; SAH, sub-arachnoid haemorrhage; 5-HT, 5-hydroxytryptamine; VIC, vasoactive intestinal contractor.

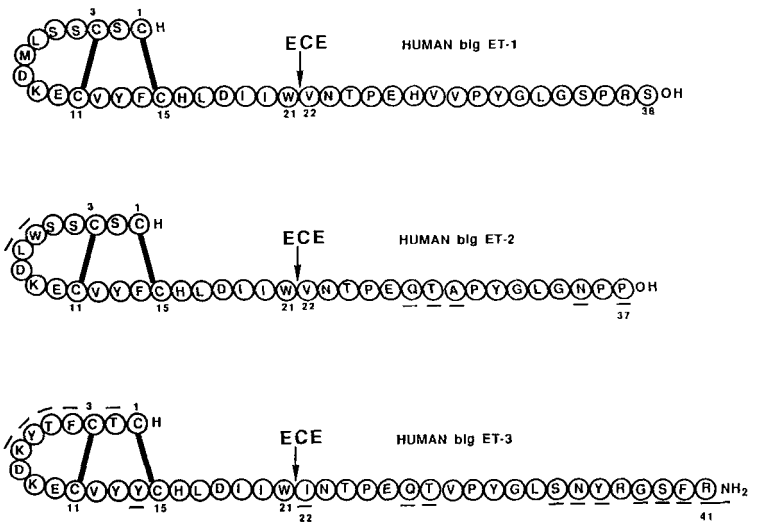

Figure 8.1. Structure of the isoforms of human big endothelin. The site of cleavage by ECE is indicated. Amino acids with bars adjacent indicate sequence changes from big ET-1.

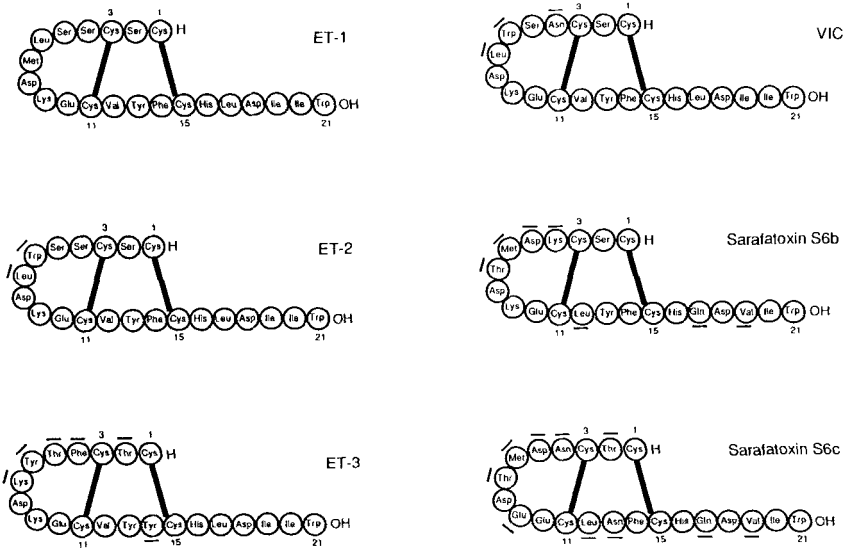

Figure 8.2. Structure of the endothelin family of peptides. Amino acids with bars adjacent indicate sequence changes from ET-1.

to the sarafatoxin snake venoms of the Israeli burrowing asp, *Atractaspis engaddensis* [3]. Yet another peptide with sequence homology to the endothelins (*Figure 8.2*) is vasoactive intestinal contractor from the mouse [4].

ET-1 is the only isoform secreted by endothelial cells. However, ET-1 and ET-3 are found in varying proportions in a wide range of tissues including brain, lung, kidney, spleen, liver, gut and bladder [5]. ET-2 has been found in a human adenocarcinoma cell line [6].

BIOLOGICAL ACTIONS OF ENDOTHELIN

The biological actions of ET-2 are, for the most part, similar to those of ET-1 but have been studied less extensively. The activity profile of ET-3 is different, this being ascribed to receptor subtype affinity (see section on endothelin receptors). The big endothelins are considerably less potent and rely to a large extent on conversion to their mature forms for activity.

CARDIOVASCULAR SYSTEM

Endothelin is a potent constrictor of isolated blood vessel preparations with EC_{50} values in the low nanomolar range. ET-1 is more potent in porcine coronary artery (EC_{50} = 7 nM) than rat aorta (EC_{50} = 20 nM) and the absence of an intact endothelium does not influence this activity [7]. Canine cerebral arteries such as the basilar (EC_{50} = 0.59 nM), posterior cerebral (EC_{50} = 0.74 nM) and middle cerebral (EC_{50} = 0.83 nM) as well as the coronary artery (EC_{50} = 0.85 nM) are particularly sensitive to ET-1 in comparison with the mesenteric artery (EC_{50} = 1.4 nM) [8]. Feline middle cerebral artery is also extremely sensitive to ET-1 (EC_{50} = 0.17 nM) and, although the potency was unaffected by endothelium removal, the maximum response was increased, suggesting an opposing endothelium-dependent dilator component [9]. Although a dilator response to ET-1 is not usually seen, low concentrations of ET-3 (below 10 nM) relax canine coronary arteries [10]. Contractile responses were seen at higher concentrations. ET-1 also contracts human isolated vessels and, for example, an EC_{50} = 5.4 nM was obtained using human omental arteries [11]. Perfused vascular beds, such as the rat mesentery, show similar effects to isolated individual vessels: ET-1 was more potent than ET-3 in increasing perfusion pressure and a decrease in pressure was more readily demonstrated with ET-3 [12–14]. In isolated vascular preparations, big ET-1 is from 10 to 300

times less potent than ET-1 [15–17]. The difference in relative potency of big ET-1 is presumably determined by the tissue ECE activity.

Endothelin also has effects on isolated cardiac preparations, although these are less marked than on vascular tissues. The positive inotropic response varies considerably between species [18]. In the most sensitive preparation, rabbit papillary muscle, there is little difference between the potency of ET-1, ET-2 or ET-3 [18]. There is also little difference between the small positive chronotropic responses to ET-1 and ET-3 [19].

In vivo, intravenous administration of ET-1 to conscious [20], anaesthetized [21] or pithed [22] rats produces a biphasic blood pressure response; a small, transient depressor response followed by a prolonged pressor response. The systemic blood pressure changes induced by ET-1 are reflected in changes in regional haemodynamics, although the dilator response is not seen in all vascular beds [23–25]. Big ET-1, when administered intravenously, is almost as potent as ET-1 in producing a pressor response which suggests effective *in vivo* conversion to ET-1 [22]. The haemodynamic effects of ET-1 have also been studied in man and pressor responses are seen after intravenous or intra-arterial administration [26–28].

Although ET-1 has positive inotropic activity *in vitro*, administration of ET-1 *in vivo* decreases left ventricular contractility and cardiac output, a result of myocardial ischaemia secondary to coronary constriction [29]. ET-1 also has pro-arrhythmic activity which may be independent of effects on the coronary circulation [30].

In addition to its acute effects on contractile function of vascular and cardiac muscle, ET-1 influences cell growth, proliferation and migration. ET-1 stimulates DNA synthesis, as indicated by increased ^{3}H-thymidine uptake [31–33], stimulates cellular Na^{+}/H^{+} exchange [33] and increases the expression of the proto-oncogenes, c-fos and c-myc [31] in vascular smooth muscle cells. ET-1 also induces cardiac gene expression [34].

OTHER SYSTEMS

Endothelin has many other biological actions, but among the most important of these are the effects on the airways, the gastro-intestinal tract, the kidney and the central nervous system. In addition to its effects on the pulmonary circulation, endothelin has marked effects on non-vascular airway smooth muscle. ET-1 is a potent constrictor of guinea-pig isolated trachea, upper bronchus and parenchyma [35]. ET-2 and ET-3 also contract isolated guinea-pig trachea, although ET-3 is less potent than the other two isoforms [36]. Endothelin contracts bronchial smooth muscle from a

number of other species including man [37, 38]. *In vivo* ET-1, intratracheally injected or dosed by aerosol, increases respiratory resistance and decreases dynamic compliance in the dog [39]. Similarly, ET-1 produces bronchoconstriction in the anaesthetized guinea-pig when administered either intravenously or by aerosol [40]. As in vascular smooth muscle cells, ET-1 is an extremely potent mitogen in airway smooth muscle cells [41]. ET-1 also increases vascular permeability in the airways (and other tissues) and the lower bronchi are particularly sensitive [42].

Endothelin not only influences the tone of GI smooth muscle [43] but also stimulates intestinal ion secretion [44]. ET-1 produces damage to the gastric mucosa although it is unclear to what extent this is a result of local vasoconstriction [45].

Many of the effects of endothelin on the CNS are probably a consequence of its profound cerebrovascular effects. However, endothelin may have a direct role in the central regulation of cardiovascular and respiratory function and also produce direct behavioural effects [46].

Similarly, renal function is markedly affected by the renovascular actions of endothelin [47]. However, the presence of non-vascular endothelin receptor sites in the kidney suggests that endothelin may modulate renal function by a direct action [48]. Indeed, a direct contractile and mitogenic action of ET-1 on mesangial cells has been demonstrated [49]. The different isoforms show different profiles in the kidney. ET-1 increases renal vascular resistance and glomerular filtration rate [50]. ET-3, however, decreases renal flow and GFR at low doses, although higher doses produce effects similar to those seen with ET-1 [51].

ENDOTHELIN INTERACTIONS

Although all the actions of endothelin described above have been considered in isolation, the interaction of endothelin with other bioactive agents may be of equal or even greater importance. Endothelin can release other agents, for example, the ET_B receptor-mediated vasodilator action of endothelin may depend on nitric oxide release as it is sensitive to N^G-nitro-L-arginine methyl ester [52, 53]. Endothelin may also release the vasodilator prostacyclin since pressor effects to endothelin are enhanced in the presence of indomethacin [54]. The effects of endothelin on vascular permeability in the rat may involve thromboxane A_2 [42] and platelet activating factor [55]. The effects of a number of agents may be mediated, at least in part, by endothelin. ET-1 synthesis is regulated by a range of cytokines in airway epithelial cells [56] and aortic endothelial cells [57], and insulin has been shown to stimulate ET-1 gene expression in endothelial

cells [58]. ET-1 can also potentiate the effects of other agents, such as the vasoconstrictor action of 5-HT [59].

ENDOTHELIN CONVERTING ENZYME

The nature of the physiologically relevant converting enzyme(s) responsible for the cleavage of the big endothelin scissile bond (Trp^{21}-Val^{22} in big ET-1 and big ET-2, Trp^{21}-Ile^{22} in big ET-3) to form endothelin, remains the subject of on-going research and has been reviewed in detail by Opgenorth *et al.* [60].

Enzymes from several different endopeptidase classes have been shown to perform this bond cleavage. Initially a chymotrypsin, serine protease, was proposed [1] and two groups have reported work on the design of inhibitors of the action of this type of enzyme (see later). It has been demonstrated, however, that a chymotrypsin-type enzyme does not cleave big ET-1 cleanly to only an active ET-1(1-21) fragment and a stable C-terminal fragment, as cleavage can occur both initially at Tyr^{31}-Gly^{32} and subsequently to break down the ET-1 formed [61].

Aspartic proteases have also been proposed as the ECE of primary interest. Enzymes of this type have been isolated from a range of tissues and have been shown to cleave big ET-1 to ET-1. The case has been proposed for pepsin, cathepsin D and cathepsin E being the important enzyme, the latter being very attractive since the human enzyme has been shown to give only ET-1 and the C-terminal fragment (22–38) as big ET-1 breakdown products [62]. However, their physiological roles continue to be questioned as they require an acidic environment for activity. Inhibitors of the aspartic protease from rat lung tissue have been reported [63] but there is no information on inhibitors of the other aspartic enzymes.

A thiol protease extracted from primary porcine aortic endothelial cells has been proposed as a potential ECE [64]. It has been shown to convert big ET-1 to ET-1 at a physiological pH of 7.0–7.5, but the C-terminal fragment (23–38) has not been identified as a cleavage product. Work is in progress to further purify the enzyme to study its actions in more detail.

The generally accepted view is that ECE is a metalloprotease. Enzymes of this type have been isolated from a variety of tissue sources including cultured endothelial cells [65], vascular smooth muscle [66], human umbilical vein [67] and human brain [68], and have been characterized as neutral endopeptidases sensitive to phosphoramidon. Several groups have demonstrated an endothelial cell derived ECE which is active at neutral pH and inhibited by phosphoramidon and other metal chelators, but not by

enkephalinase inhibitors such as thiorphan or ACE inhibitors such as captopril [69, 70]. Although metalloproteases have generally been reported as membrane bound ECEs they have been found in both membranous and cytosolic fractions of cultured bovine and porcine endothelial cells [71].

Phosphoramidon has now been shown to inhibit endothelin production by endothelial cells exposed to cyclosporin [72] and to abolish the increases in ET-1 release which can be induced by ischaemia-hypoxia in isolated perfused guinea-pig lungs [73].

In vivo, phosphoramidon has been shown, along with the ET_A receptor antagonist BQ123, to lower blood pressure in conscious spontaneously hypertensive rats [74] and to inhibit big ET-1-induced sudden death in mice [75].

ECE INHIBITORS

The search for inhibitors of ECE activity is the subject of research by many groups, but published information on progress in this endeavour is only just beginning to appear. The first claims to the identification of ECE inhibitors came from Abbott Laboratories with the publication of a range of compounds which inhibited an aspartic protease from rat lung tissue [63]. This series of statine-containing analogues showed high selectivity over renin and cathepsin D and had an IC_{50} of approximately 3 nM (1), (2). However, there are now doubts that this pepstatin A-inhibitable enzyme is the main ECE of pharmacological relevance, with the evidence now suggesting this to be a metalloprotease. An approach, undertaken by Farmitalia Carlo Erba, is the identification of peptide inhibitors of α-chymotrypsin proteolysis of big ET-1 [79]. These peptides are claimed to bind to the internal segment of big ET-1 (i.e. 13–30), thus inhibiting ET-1(1–21) formation. Therefore, they inhibit the initial proteolytic step which is the cleavage of the terminal of big ET-1(32–38). This then stops any

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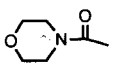
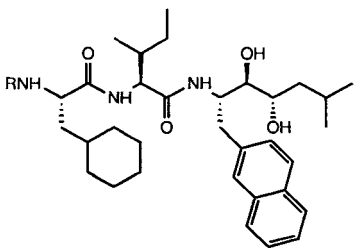
(2) R = 



subsequent cleavage to ET-1 at Trp^{21}–Val^{22}. The peptides, claimed to bind to human big ET-1, are up to twenty amino acid residues in length and contain only D-residues. The most potent examples (Asbig-1 and Asbig-2) show this activity at 23.1 μM using an equivalent concentration of big ET-1 (3), (4).

H-Arg-Asn-Asn-Val-Leu-Gly-Ser-Val-Asp-Pro-Asn-Asp-Val-His-Val-Ala-Glu-Val-OH

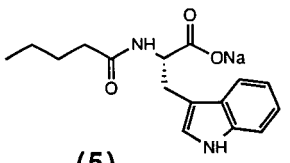
(3) Asbig 1

H-Gly-Asn-Asn-Met-Leu-Gly-Ser-Val-Asn-Pro-Asp-Asp-Ile-Glu-Met-Pro-Lys-Val-OH

(4) Asbig 2

Zamai *et al.* from the same company have also published data on sequence-directed peptide inhibitors [80]. They used the model of big ET-1 which they proposed on the basis of computerized structure prediction, molecular mechanics minimization and molecular dynamics [81]. The effect of the sequence-directed inhibitors on the *in vitro* chymotrypsin-catalyzed hydrolysis of big ET-1 was investigated as a strategy for inhibiting formation of endothelin.

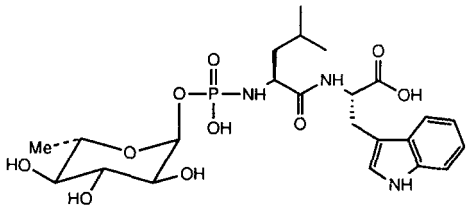
In terms of smaller molecules, there is a claim that n-pentanoyl-tryptophan (5) inhibits the vasoconstrictive effects of endothelin, but no details of biological activity are quoted [82].



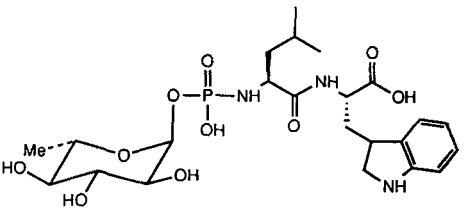
(5)

The work carried out to isolate and identify the pharmacologically important ECE has characterized the enzymes as metalloproteases because of their inhibition by the known metalloprotease inhibitor phosphoramidon (6). Many groups have published data on the use of phosphoramidon in their enzyme systems. A patent application for phosphoramidon and its close analogues for use as an endoserine – (endothelin) – converting enzyme

inhibitor has been filed [83]. The indoline analogue (7) of phosphoramidon has also been cited as an ECE inhibitor [84].



(6)

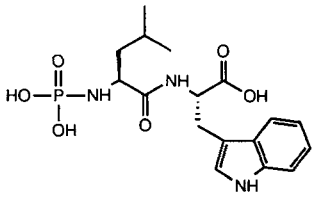


(7)

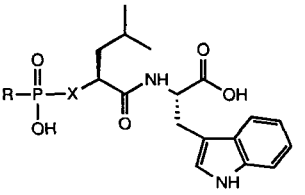
A recent publication from Pollock *et al.* [85] has shown that the rhamnose group in phosphoramidon is not responsible for its inhibition of the response to big ET-1 in the rat, as the analogue lacking this group (8) demonstrated equivalent activity in their model on continuous infusion. Analogues of phosphoramidon where the rhamnose group has been replaced with alkyl or alkoxy groups have been described by Bertenshaw *et al.* [86]. The most potent of these compounds (9) had an $IC_{50} = 2\ \mu M$ in a rabbit lung ECE screen, but interestingly the equivalent compounds with the NH group replaced by O or CH_2 (10,11) showed no activity.

A series of more potent phosporamidate derivatives has been described by Takeda researchers [87]. One of these examples (12) shows inhibition of the ECE from pig lung with an $IC_{50} = 0.1\ \mu M$, a considerably lower value than that for phosphoramidon which is found to have an IC_{50} in the 1 to 25 μM range. The identification and use of compounds with this level of potency should facilitate further research into the utility of inhibitors of ECE.

A recent trend that has now spread to the endothelin area is the patenting of enzymes themselves and the use of them in test systems. The first patent application of this type appeared from Berlex in August 1992 [76]. The theme of this patent was the substantial purification of ECE from cells

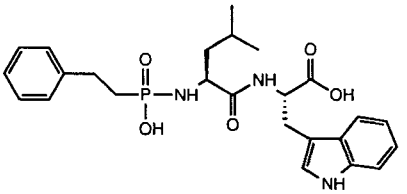


(8)



X = NH (9), O (10), CH_2 (11)

R = ALKYL or ALKOXY



(12)

derived from human lung tissue. The characterization was of a protein that cleaved big ET-1 to yield ET-1, was sensitive to phosphoramidon and EDTA, and was activated by chloride ions. The size estimated from gel filtration was 400 kDa. Several SDS-PAGE molecular weights were quoted for various ECE preparations; 205 kDa, 140 kDa and 100 kDa.

Two patents have also appeared from Nisshin Flour Milling Co. which claim the ECE derived from human placental tissue. One claims an enzyme which cleaves big ET-1 between the residues 21 and 22, has an optimum pH of 6.5 to 7.5 and a molecular weight of around 100 kDa, 240 kDa or above 500 kDa [77]. The other patent also claims an enzyme which cleaves big ET-1 to ET-1 but this has an optimum pH of 3.5 to 4.5 and a molecular weight of approximately 85 kDa [78]. Whether any of these patents are granted remains to be seen, but if they are, there will be obvious repercussions for other groups working in the ECE area.

ENDOTHELIN RECEPTORS

Endothelin receptors have been classified in terms of their affinity for the

endothelin isoforms. A receptor subtype with the affinity order ET-1 = ET-2 > ET-3 has been cloned and designated ET_A [88]. A second subtype with affinity order ET-1 = ET-2 = ET-3 has also been cloned and designated ET_B [89]. This classification has been further supported by the discovery of other subtype-selective ligands. For example, sarafatoxin S6c is a potent agonist at the ET_B receptor but a poor agonist at the ET_A receptor [90]. Similarly, [$Ala^{1,3,11,15}$]ET-1, ET-(16-21), BQ 3020 and IRL 1620 are selective ET_B subtype agonists [91–94]. [Formyl-Trp^{21}]ET-1 appears to be a selective ET_A subtype agonist [95]. The selectivity of antagonists is described in the next section.

ET_A and ET_B receptors are widely distributed and numerous ligand-

Table 8.1. LOCATION AND FUNCTION OF ET RECEPTOR SUBTYPES

Location	*Function*	*Reference*
ET_A receptors		
Guinea-pig aorta	Contraction	[96]
Guinea-pig pulmonary artery		[97]
Rat aorta		[98]
Rat intracerebral artery		[99]
Rabbit carotid artery		[100]
Rabbit renal afferent arteriole		[101]
Pig coronary artery		[102]
Dog coronary artery		[10]
Dog basilar artery		[103]
Human saphenous vein		[104]
Rat aortic smooth muscle cells	Mitogenesis	[105]
Rat heart	Extravasation	[106]
Rat vas deferens	Neurotransmission potentiation	[107]
ET_B receptors		
Rat aorta endothelium	Vasorelaxation	[108]
Dog coronary artery endothelium		[10]
Pig pulmonary artery endothelium		[93]
Pig pulmonary artery	Contraction	[109]
Pig pulmonary vein		[109]
Pig coronary vein		[109]
Pig coronary artery		[102]
Rabbit pulmonary artery		[93]
Rabbit saphenous vein		[100]
Guinea-pig ileum		[110]
Guinea-pig trachea		[97]
Guinea-pig bronchus		[96]
Rat jugular vein		[98]

selective binding sites have been demonstrated. However, many of these have yet to be associated with a functional response. A selection of locations at which a functional response for ET receptor subtypes has been demonstrated is shown on *Table 8.1.*

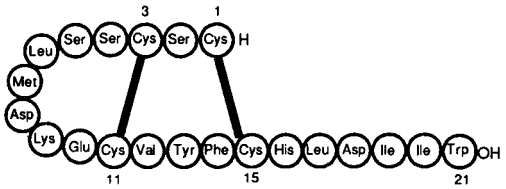
Claims for further receptor subtypes have been made. A third subtype, ET_C, with highest affinity for ET-3, and which mediates inhibition of prolactin release from the anterior pituitary, has also been proposed [111]. More recently, cloning of an ET_C receptor, from *Xenopus laevis* dermal melanophores, has been reported [111a]. An ET receptor in pig coronary artery recognises ET-3 and sarafotoxin S6c but not ET-1 or sarafotoxin S6b [112]. A 'super-high' affinity version of the ET_B receptor has also been claimed in rat brain and atrium [113]. The ET receptor which mediates contraction of the rat left atrium may also be different as it cannot be readily classified as ET_A or ET_B [114]. The ET receptor in rabbit pulmonary artery and rat stomach has ET_B characteristics in terms of agonist sensitivity, yet it is resistant to the mixed ET_A/ET_B antagonist, PD 142893 (16) [115]. It remains to be confirmed whether these examples constitute genuine new receptor subtypes.

ENDOTHELIN RECEPTOR ANTAGONISTS

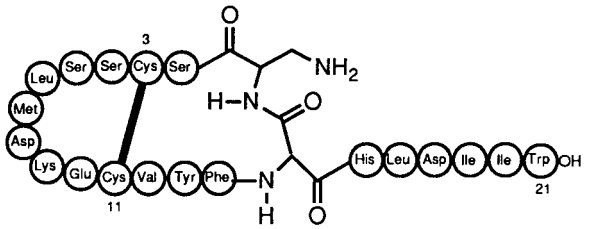
ENDOTHELIN ANALOGUES

The initial chemistry carried out around the endothelin and sarafotoxin structures, by modification of the amino acid sequences, generated a range of peptide agonists. These compounds, with a wide range of relative potencies, have been used to probe the essential structural features of the natural pharmacologically active agents.

Further modification of the structures has now also given rise to an increasing range of antagonists. A synthetic peptide homologue of ET-1 (13) but with an amide link replacing the disulphide [1, 15] (14) has been shown, by Anderson *et al.*, to block the vasoconstrictor effects of ET-1 in guinea-pig lung vascular tissue [116]. This compound [Dpr^1,Asp^{15}] ET-1, in which the Cys^1 was replaced by diaminopropionic acid, and Cys^{15} by aspartic acid, linking to form the amide bond, did not alter the pulmonary vasoconstriction induced by thrombin, adrenaline or ET-3, and hence was specific to ET-1 [117]. [Dpr^1,Asp^{15}] ET-1 and analogues with Glu^{15} and other replacements in the sequence of ET-1(1-21) are the subject of a patent from Research Corporation Technologies which gives an IC_{50} for (14) as 2 nM in guinea-pig lung tissue [118].



ET-1 **(13)**



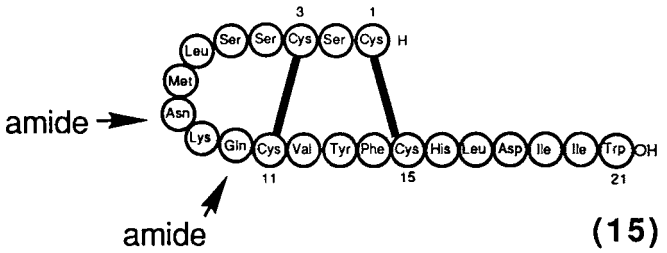
(14)

A sequential D-amino acid and L-Ala replacement in ET-1 has also shown that antagonism can be found, especially for [Ala[8]] ET-1 and [D-Met[7]] ET-1 but receptor selectivity was not described [119].

Takeda researchers have shown that antagonist activity is present in a series of ET-1 analogues [120]. Results from a porcine myocardial membrane binding assay, and from antagonist activity against ET-1 constricted porcine coronary artery, suggest that [Thr[18] Leu[19]] ET-1 and [Thr[18] Cha[19]] ET-1 are the most potent analogues (pA_2 = 7.7 in the latter model) although other modifications also gave antagonists.

Antagonist activity for a range of ET-1 and sarafatoxin analogues has been demonstrated in a rat brain membrane binding assay [121]. The preferred compounds in this series were [Gly[7]] ET-1 and [Thr[2],Val[12],Leu[17],Ile[19]] S6b. These analogues had IC_{50} values of 1.4 nM and 1.3 nM, respectively. No data on receptor selectivity or in functional assays is available.

ET-1 with the acidic residues at 8 and 10 converted to the corresponding



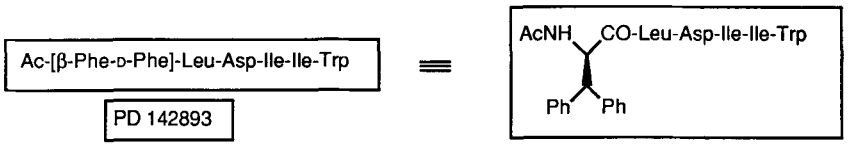
(15)

amides (15) is also claimed as having antagonist activity [122] but again no further data is disclosed.

The ET-1 C-terminal hexapeptide ET(16–21) has been shown to have agonist activity in the guinea-pig isolated bronchus (ED_{50} = 0.228 μM) but truncated versions show greatly reduced potency [123]. Modification of the hexapeptide has led to several series of compounds with antagonist activity. One of the first compounds of particular interest was PD 142893 (16). This has been shown to have similar [^{125}I] ET-1 binding affinities in membrane preparations derived both from tissues which contained mainly ET_A receptors (rat left ventricle (IC_{50} = 29.0 nM) and rabbit aorta (IC_{50} = 80.7 nM)) or mainly ET_B receptors (rat cerebellum (IC_{50} = 46.5 nM) and rabbit pulmonary artery (IC_{50} 36.7 nM)). It has also shown non-selective antagonism in functional bioassays which used rabbit isolated renal artery (ET_A) and rabbit pulmonary artery (ET_B) [124]. Interestingly, PD 142893 appeared to show selective ET_B antagonism in the anaesthetized rat [124]. Further work, substituting Phe for Asp in PD 142893, gave a compound (17) which now showed selectivity for ET_B receptors *in vitro* [125]. An analoguous compound, PD 145065 (18), described in a Parke-Davis /Warner Lambert patent [126] is another nonselective antagonist and has been shown to block the ET-1-induced vasoconstrictor effects in the rat kidney. This is by a non-selective action, both *in vitro* and *in vivo* [127].

Selective ET_B antagonist properties have been claimed in a Banyu patent for at least one example, la (19) in a series of linear ET-1 analogues (IC_{50} = 1300 nM against ET_A and 1.1 nM against ET_B, in binding assays) [128] but no information from functional assays is available.

The first compound with published detailed information which supports the claims for a specific ET_B antagonist is IRL 1038, (20) [Cys^{11}-Cys^{15}] ET-1 (11-21). This compound showed specific antagonist activity both in binding assays and in endothelin-induced contraction models. With ET-3 as agonist, 3 μM IRL 1038 antagonized the ET_B receptor-mediated contraction of guinea-pig ileal and tracheal smooth muscle but did not affect the ET_A receptor-mediated contraction of rat aortic tissue [110].



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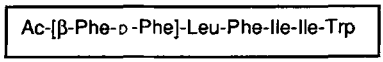

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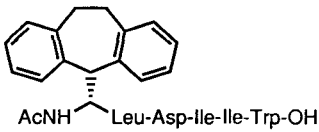
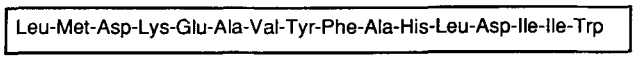


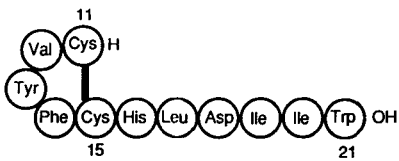
PD 145065 **(18)**

PEPTIDE ANTAGONISTS FROM NATURAL PRODUCTS

Most of the other compounds which have been discovered to have endothelin antagonist properties were identified as a result of the random screening of natural products in endothelin binding assays.

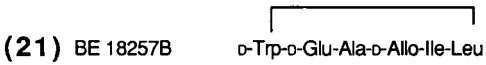


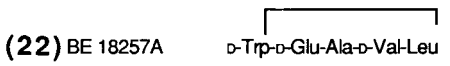
Banyu 1a **(19)**



IRL 1038 **(20)**

The first compound reported as an antagonist, whose structure was not derived from the natural endothelin structure, was identified by workers at Banyu as a cyclic peptide, BE 18257B (21). This compound was isolated from the fermentation products of *Streptomyces misakiensis*, BA 18257. It is a novel cyclic pentapeptide, cyclo(-D-Glu-L-Ala-allo-D-Ile-L-Leu-D-Trp-) [129, 130]. It showed relatively weak activity (IC_{50} = 1.4 μM in porcine aortic smooth muscle) but was highly selective for ET_A receptors and antagonized ET-1-induced vasoconstriction in the rabbit iliac artery. The taxonomy, fermentation, isolation and characterization of BE 18257B and its analogue BE 18257A (22) have been described together with the structure-determination [131, 132].

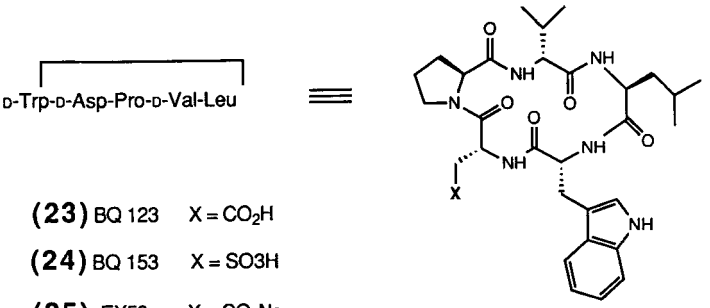




Synthetic work, based on these initial compounds, has now produced novel analogues which demonstrate more potent selective endothelin antagonism [102, 133]. The compounds of greatest interest are BQ 123 (cyclo(-D-Trp-D-Asp-Pro-D-Val-Leu-)) (23) and BQ 153 (cyclo(-D-Trp-D-Sal*-L-Pro-D-Val-L-Leu-)) (*Sulfoalanine) (24) which show greatly improved binding affinity for ET_A receptors on vascular smooth muscle cells (IC_{50} = 7.3 and 8.6 nM, respectively), and very poor binding to ET_B receptors in cerebellar membranes (IC_{50} = 18 and 54 μM). BQ 123 and BQ 153 also antagonized ET-1-induced constriction of isolated porcine coronary artery (pA_2 = 7.4).

BQ 123 has sufficient aqueous solubility for intravenous administration. This property, its potency and selectivity have led to its widespread use as the first standard in the endothelin antagonist field. It is used by many groups as the main tool in research into the distribution of endothelin receptor subtypes [134, 135]. Studies have also been reported using the sodium salt of BQ 123 (EX59) (25) [136].

Apparently concurrent with this work, a closely analogous series



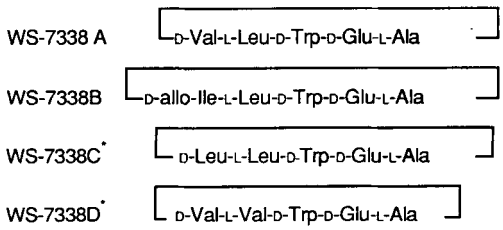
(23) BQ 123 $X = CO_2H$

(24) BQ 153 $X = SO3H$

(25) EX59 $X = CO_2Na$

of compounds was discovered by Fujisawa scientists. The first compounds (26) were isolated by culturing Streptomyces sp. No. 7338 [137]. These compounds were designated WS-7338A, B, C, D. The most potent, WS-7338B (cyclo(-D-Trp-D-Glu-L-Ala-D-allo-Ile-L-Leu-)), had an IC_{50} = 0.27 μM against ET-1 in porcine aorta, an IC_{50} = 0.48 μM against ET-2, but there was no effect on ET-3 binding in this tissue. Again the taxonomy, fermentation, isolation, characterization and pharmacology have been reported [138, 139].

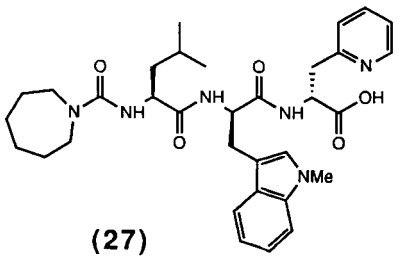
Conformational studies have been carried out on BQ 123 [140, 141] and WS-7338B (also described as BQ 518) [142] and subsequent medicinal chemistry has resulted in series of novel linear tripeptides from both Banyu and Fujisawa [143, 144].



(26) *Structures of C and D are the tentative structures

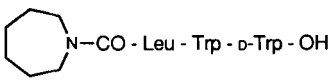
The preferred Fujisawa compound, FR 139317 (2(*R*)-[(*R*)-2-[2(*S*)-[[[1-(hexahydro-1*H*-azepinyl)]carbonyl]amino]-4-methylpentanoyl]amino]-3-[[3-(1-methyl-1*H*-indolyl)propanoyl]-amino]-3-(2-pyridyl)propanoic acid) (27)

is understood to be ready to begin clinical trials [145]. Efficacy has been claimed, for the injectable form, in experimental models of cerebrovascular spasm following sub-arachnoid haemorrhage, ischaemic heart disease, pulmonary hypertension, and renal failure. The preferred indication for clinical investigation is not known.



(27)

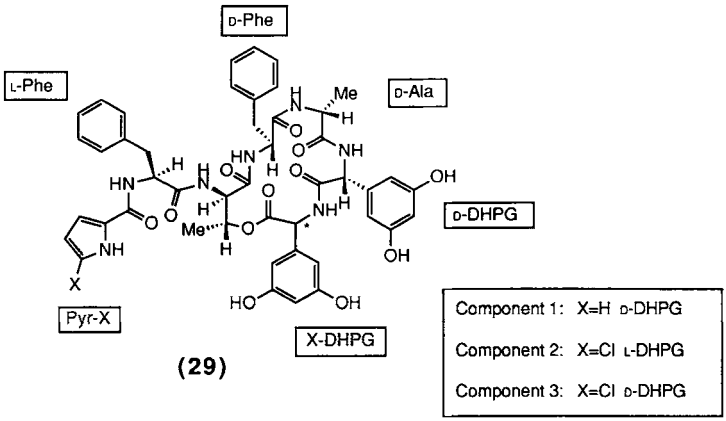
A Banyu patent, which describes 203 compounds, highlights a very similar compound (example 50) (28) which is reported to show 87% inhibition at 1.1 μM of [^{125}I] ET-1 binding in porcine aorta receptors, and activity *in vitro* in the guinea-pig trachea and on rat heart perfusion pressure. There is no other published information on this series of compounds.



(28)

Three novel cyclic depsipeptides have been isolated from cultures of Microbispora sp. MA6857, ATCC 55140. These compounds (29) demonstrate endothelin antagonism in [^{125}I] ET-1 binding assays using rat aorta (ET_A) and rat hippocampus (ET_B). They are not reported to show significant selectivity between ET_A and ET_B receptors [146]. Also from a natural product source is substance WF13890 which has been isolated from a culture of Hyaldodendron sp. microbe, but its characterization has not yet been reported. It is claimed as an endotherin (endothelin) antagonist for use in ischaemic disease [147].

These small peptides provide starting points for the development of more potent and selective analogues. Molecular modelling studies in particular should provide suggestions for chemical modifications which could lead to



(29)

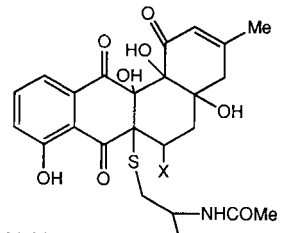
more useful tools for investigating the role of endothelin and ultimately to the identification of drug candidates.

NON-PEPTIDE ANTAGONISTS

Natural products have also proved to be the source of some of the increasing number of reported non-peptidic endothelin antagonists. These compounds have generated a great deal of interest as they provide leads for further development in the attempt to identify potent selective or non-selective agents to ascertain whether antagonists of the action of endothelin will be useful pharmacological agents.

A series of anthraquinone antagonists was isolated by Fujisawa workers from fermentations involving Streptomyces. This was carried out from Streptomyces sp. No. 89009 (FERM BP-2475) and also from Pseudomonas cepacia No. 97 (FERM BP-2945) [148–150]. The most active compounds (R = H) FR901366 (WS 009 A) (30) and (R = OH) FR901367 (WS 009 B) (31) showed binding in porcine aorta at 5.8 μM and 0.67 μM, respectively and FR901367 showed activity in the rat at 10 mg/kg i.v. against ET-1-induced pressor responses. No information on receptor subtype selectivity is available for this series.

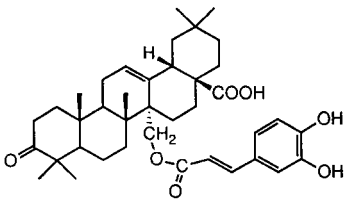
A series of selective non-peptide ET_A receptor antagonists has also been reported by scientists at Shionogi [151]. The initial compound in this series, myriceron caffeoyl ester 50-235, (32), was isolated from the bark of the bayberry, *Myrica cerifera* [152]. It has been shown to displace [^{125}I] ET-1 binding, in rat cardiac membranes, with a K_i value of 78±18 nM, but at 1 μM 50-235 inhibited only 25% of [^{125}I] ET-3 binding. In the rat thoracic



(30) X = OH

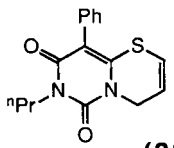
(31) X = H

aorta, 50-235 antagonized ET-1-induced vasoconstriction with a pA_2 value of 6.65 ± 0.13 and demonstrated its specificity as it had no effect on the contraction induced by potassium chloride or adrenaline.



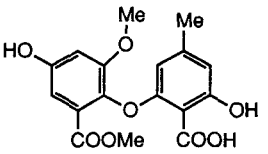
(32)

An early patent from Takeda described a series of sulphur-containing fused pyrimidines as 'useful for treatment and prevention of diseases induced by abnormalities in regulation of reactions *in vivo* mediated through endothelin or interleukin1' [153]. The examples, with data for endothelin inhibition by (33), showed activity in porcine coronary arteries against endothelin-induced contraction. The most active compound gave 82.3% inhibition at 1 μM. Interesting activity was found both orally and intravenously in the conscious dog against endothelin-induced pressor and depressor responses, and orally at 100 mg/kg in a model of infarct size in the rat heart.



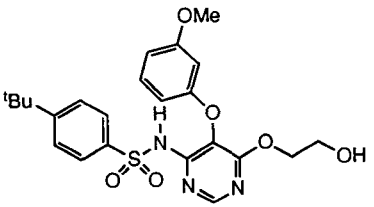
(33)

Another publication from Takeda described a series of asterric acid analogues as endothelin antagonists. These compounds, TAN-1415 derivatives, have been patented for the indications of myocardial infarction or renal ischaemia [154]. The only reported activity of the asterric acid itself and close analogues (34) is that they showed inhibition against pressor effects, induced by ET-1 in the rat, at 50 mg/kg i.v.



(34)

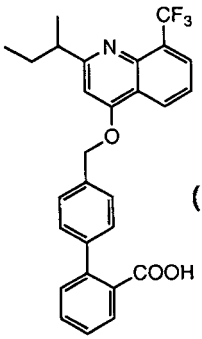
A series of sulphonylaminopyrimidines has been reported by workers at Hoffman-La Roche [155, 156] to demonstrate antagonist activity in a human placental binding assay, the preferred example (35) showing an IC_{50} = 0.2 μM. These compounds had previously been described as hypoglycaemic agents, but with the level of potency shown as endothelin antagonists, could provide an interesting starting point for further chemical development.



(35)

Claims that compounds with activity in the angiotensin area also inhibit the actions of endothelin have appeared in the literature [157]. The only compounds patented for both these properties are three series of compounds from Roussel-Uclaf [158–160]. Details of activity are shown for only one compound (36) which has very weak activity, IC_{50} = 14,000 μM in the rat cortical membrane binding assay. It is claimed to be active at 1 mg/kg i.v. in the rat against an ET-1-induced vasoconstriction [160] which could

indicate that there is some overlap in the structural types of interest in the two receptor systems.



(36)

Thus, endothelin antagonists with a wide range of chemical structures are emerging, and these may prove to be useful tools to explore the physiological role of endothelin.

ENDOTHELIN ANTIBODIES

Endothelin antibodies have been developed by several groups, and have been used to set up sensitive radio-immunoassays (RIA) for the investigation of the function and mechanism of endothelin actions. Having developed monoclonal and polyclonal antibodies against ET-1, Saito *et al.* used them to show that endothelial cells secrete big ET-1 as well as ET-1 [161]. This study further demonstrated the existence of ET-1-like immunoreactivity in human plasma and raised the possibility that endothelin is a circulating hormone as well as a local one but did not define the precise nature or source of these compounds in human plasma. The RIAs that they subsequently developed have been used to detect raised levels of ET-1-like immunoreactivity in patients with essential hypertension [162]. The pathophysiological significance of this finding still needs to be investigated.

Studies in animal models using endothelin antibodies are now being reported. Miyamori *et al.* demonstrated in rabbits [163] that endothelin antibody administration failed to show any appreciable changes in systemic or renal haemodynamic parameters suggesting that endothelin in the circulation may not contribute to blood pressure maintenance under normal conditions. However, its role as a local modulator is as yet not defined.

In spontaneously hypertensive rats, endothelin has been shown, by studies using endothelin antibodies, to play an important role in the modulation of systemic blood pressure and renal function [164], but there were no significant effects in Wistar Kyoto rats which may therefore be less sensitive to endothelin [165].

Studies in the rat by Morales *et al.* have shown that intravenous injections of endothelin antibody dose-dependently decreased the haemorrhagic erosions caused by ethanol in a model of gastric injury. This suggests that endothelin may be an endogenous mediator in the pathogenesis of gastric mucosal lesions induced by exogenous agents [166].

Information on receptors, using antibodies, was first provided by Kondoh *et al.*. This group prepared monoclonal antibodies which were reactive with endothelin receptors by the immunization of mice with rat lung membranes [167]. Four clones were isolated and these demonstrated that there was more than one receptor as they exhibited different affinities to two ET receptors. One of these receptors was of 32 kDa and the other 45 kDa.

More recently, monoclonal and polyclonal antibodies to ET-1 have been generated by Traish *et al.* which bound ET-1 specifically and with high affinity even at dilutions of $1:10^6$ [168]. The monoclonal antibodies cross-reacted with ET-2, VIC, big ET-1 and ET-3 with varying affinity. The results suggest that the binding site lies in the N-terminal region of endothelin, and suggests more precisely the 8–16 amino acid region as being of most interest. The antibodies were deemed specific to endothelin as they did not cross-react with other unrelated peptides. They have been used to demonstrate the presence of immunoreactive endothelin in the endothelial lining and in the smooth muscle of blood vessels.

The increasing availability of antibodies should facilitate studies into whether or not ET-1 is synthesized and released at or near the site of action and make it possible to determine the levels of endothelin in pathological tissue samples and correlate this to the state of a disease.

BIOLOGICAL TEST SYSTEMS

TESTS FOR ECE INHIBITORS

ECE inhibitors can be evaluated in enzyme assays, isolated tissues and whole animals. A range of tissues (for example, vascular endothelium, vascular smooth muscle, lung) demonstrate phosphoramidon-sensitive ECE activity and provide a choice of starting points for an enzyme assay for ECE inhibitors. Partially purified ECE, such as that from rabbit lung, is

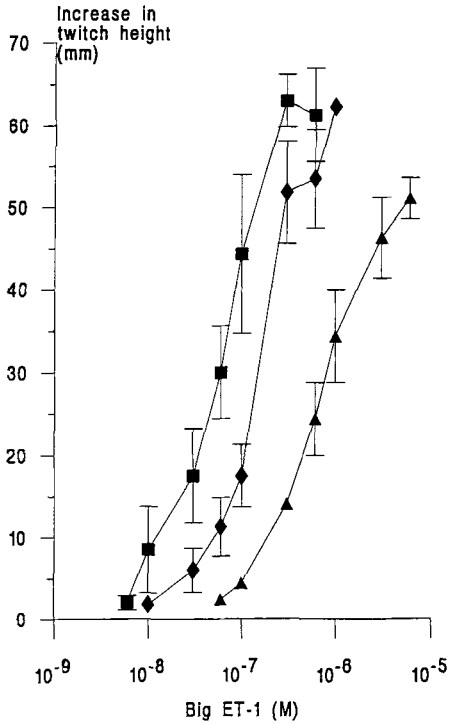


Figure 8.3. Concentration response curves to big ET-1 for the potentiation of twitch height in rat isolated vas deferens in the absence (squares) and in the presence of phosphoramidon 10 μM (diamonds) and 100 μM (triangles). Values are mean, n = 4–8, and vertical bars represent S.E.M. Data provided by S. Stoggall.

normally used and big ET-1 is the substrate [86]. In this system, phosphoramidon, EDTA and 1,10-phenanthroline are inhibitors, thiorphan is a very weak inhibitor and captopril and kelatorphan are inactive. Measurement of the ET-1 product can be made by commercially available RIA or by HPLC using either u.v. or radiochemical (^{125}I) detection. Alternatively, a fluorescence assay using succinyl-Ile-Ile-Trp-methyl-coumarin amide as substrate has been described [169].

A phosphoramidon-sensitive ECE has been identified by functional studies in rat vas deferens [170] and therefore this is a potential isolated tissue screen for testing ECE inhibitors. Big ET-1, 3-600 nM, increases the twitch height to electrical field stimulation in the isolated vas deferens preparation and this effect is inhibited in the presence of phosphoramidon

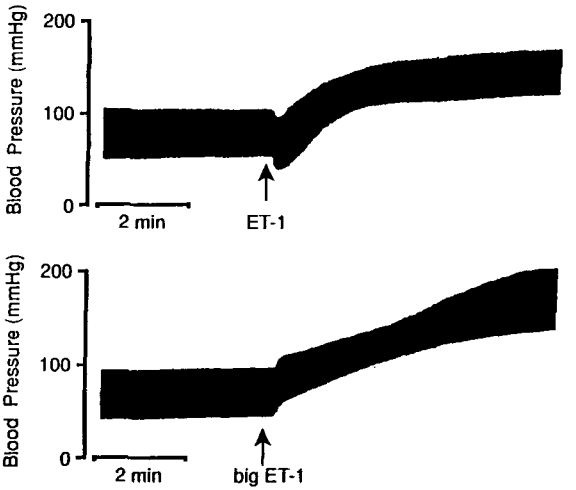


Figure 8.4. Typical tracings of the blood pressure responses to bolus i.v. administration of ET-1 and big ET-1, both 1 nmollkg, in the pithed rat. Adapted from Slee et al. *[22].*

(*Figure 8.3*). Thiorphan is less effective than phosphoramidon and enalapril is inactive. Thus, there is an apparent correlation between the ECE studied in the enzyme assay and that in the isolated tissue.

The pressor response to big ET-1 can be used as the basis for an *in vivo* test for ECE inhibitors. In the pithed rat, an intravenous bolus dose (1 nmol/kg) of big ET-1, produces a marked and sustained rise in arterial pressure (*Figure 8.4*) which can be inhibited in a dose-related manner by 1 minute pre-treatment with a bolus dose of phosphoramidon, 1–10 mg/kg i.v. (*Figure 8.5*).

TESTS FOR ENDOTHELIN RECEPTOR ANTAGONISTS

As with ECE inhibitors, a cascade of tests increasing in complexity can be assembled for ET receptor antagonists. At the cellular level, affinity of ligands for the ET_A receptor can be assessed by measuring inhibition of [^{125}I] ET-1 binding to rat A10 cell membranes using a commercially available kit. An estimate of ET_B receptor affinity can be obtained by another commercially available kit which uses human placental cell membranes (subtype ratio ET_B : ET_A = 4 : 1). Alternatively, molecular biology techniques allow the cloning of the receptor of choice and expression in a suitable cell system, such as BHK [171] or COS [172] cells, for use in binding studies.

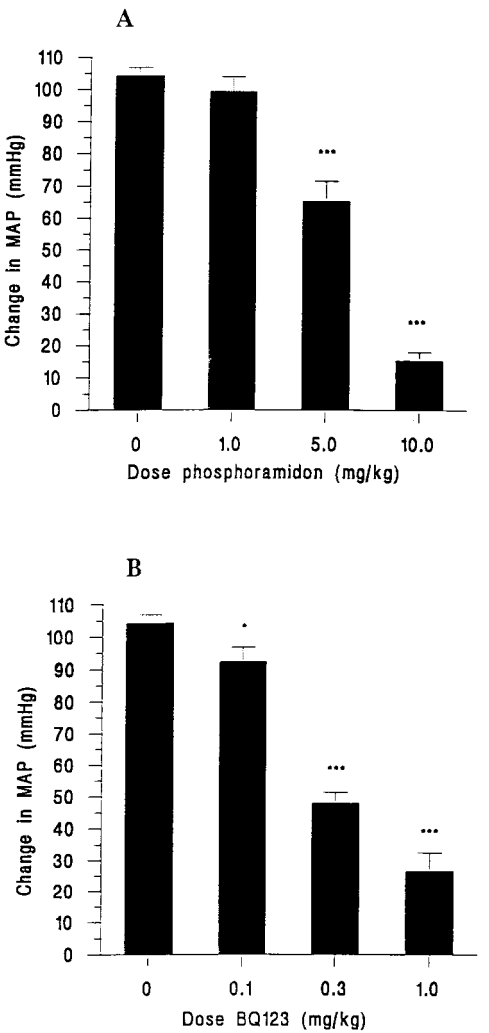


*Figure 8.5. The effect of i.v. administration of A. phosphoramidon and B. BQ 123 on the pressor response to a bolus dose of big ET-1, 1 nmol/kg i.v.. Values are mean, n = 4–10, and S.E.M. are shown. Significant differences from control are represented by *, $P<0.05$ and ***, $P<0.001$. Data adapted from Slee* et al. *[22].*

Functional ET_A receptor antagonism can be tested in an isolated blood vessel preparation. The guinea-pig and rat aorta and pig coronary artery

contract readily to ET-1. ET-1 concentration-response curves are shifted to the right in the presence of the ET_A-selective antagonist BQ 123 to yield pA_2 values in the range 6.9–7.4 [96, 98, 102]. Isolated blood vessels can also be used to screen for ET_B receptor antagonists. Endothelium-intact pig intrapulmonary arteries pre-contracted with 1 μM noradrenaline relax in response to ET_B receptor agonists such as [$Ala^{1,3,11,15}$]ET-1 [93] and can thus be used to test for antagonism.

The pressor response to an intravenous bolus dose of ET-1 (1 nmol/kg) in the pithed rat is preceded by a transient depressor response (*Figure 8.4*) which complicates the interpretation of data obtained with antagonists. This problem can be avoided by using big ET-1. The pressor response to big ET-1 can be antagonized in a dose-related manner by 1 minute pre-treatment with a bolus dose (0.1-1 mg/kg i.v.) of BQ 123 (*Figure 8.5*). The depressor responses to ET-1 can be used as the basis of an *in vivo* ET_B antagonist screen. More pronounced depressor effects are seen in anaesthetized, rather than pithed, rats where the resting blood pressure is higher, and also where a selective ET_B receptor agonist is used [24].

THERAPEUTIC TARGETS

Numerous therapeutic targets have been proposed for inhibitors of the action of endothelin but, as yet, none have been established definitively. Many of the targets have been postulated because increased tissue or plasma levels of endothelin have been detected in disease states. However, such information does not allow us to identify whether increased endothelin levels are the cause, or result, of the disorder. More convincing are the studies in which inhibitors of the actions of endothelin have been used.

Most interest has centred on cardiovascular disease and there appears to be strong evidence for a role of endothelin in a number of circulatory disorders. Of the non-cardiovascular indications, asthma may offer the greatest potential.

CARDIOVASCULAR DISEASE

The potent, prolonged vasoconstrictor effects of endothelin (particularly ET-1), in addition to its mitogenic action, constitutes the ideal profile for a potential pathological role in the cardiovascular system. Thus, in disease states where elevated local or plasma levels of endothelin have been found, the question of whether endothelin plays a causative role has been addressed.

Cerebrovascular disease (stroke)

Endothelin has been proposed as a mediator of both haemorrhagic and ischaemic stroke, although there is considerably more evidence to support the former. Cerebral vasospasm often occurs after sub-arachnoid haemorrhage (SAH) and is responsible for high rates of morbidity and mortality among survivors of the initial haemorrhage [173]. The effects of endothelin on the cerebral vasculature mimic the profile seen in cerebral vasospasm, namely, an unusually prolonged vasoconstriction which is not overcome by cerebral autoregulation [174]. Endothelin levels are elevated in the plasma of SAH patients and peak endothelin levels correspond with onset of vasospasm [175]. Similarly, elevated endothelin levels have been found in the cerebrospinal fluid of SAH patients who experience vasospasm [176]. In animal models of SAH, an endothelin antibody AwETN40 [177], the ECE inhibitor phosphoramidon [178] and the ET_A-receptor antagonist FR139317 [179], have demonstrated anti-vasospastic activity. Oxyhaemoglobin, released by decaying erythrocytes, has also been proposed as the mediator of cerebral vasospasm [180]. However, it has been demonstrated that oxyhaemoglobin releases endothelin [181] and that the actions of oxyhaemoglobin can be attenuated by BQ 123 [182].

Endothelin may also have a role in ischaemic stroke. Endothelin, released after ischaemia caused by thrombo-embolic occlusion of a cerebral vessel, could propagate further infarction by constricting the collateral circulation. Much less information is available on endothelin in ischaemic stroke but endothelin levels are raised in animal models of focal ischaemia [183] and in ischaemic stroke patients [184].

Ischaemic heart disease

Endothelin has been implicated in myocardial infarction, coronary vasospasm and re-stenosis subsequent to percutaneous transluminal coronary angioplasty. The potent, prolonged coronary vasoconstrictor and mitogenic actions of endothelin are well-documented (see earlier section) and thus endothelin is certainly able to produce the biological effects seen in these disease states. Although a reduction in infarct size has been demonstrated in a rat model of myocardial infarction with an endothelin antibody [185], and with phosphoramidon [186], the argument for a role for endothelin in ischaemic heart disease has been based mainly on the finding of increased plasma endothelin levels in patients with myocardial infarction [187–190], coronary vasospasm [191, 192] and re-stenosis post-PTCA [193–195].

Acute renal failure

Endothelin may be involved in acute renal failure of various aetiology. High levels of urinary endothelin (ascribed to renal production and not renal clearance) have been found in patients with acute renal failure resulting from a range of different causes, with levels later returning towards normal in survivors of the acute episode [196]. The three causative factors to which endothelin has been most frequently linked are ischaemia, immunosuppressant drugs and radiocontrast media (RCM). An endothelin antibody has shown marked protection against the fall in renal blood flow and GFR in a rat model of post-ischaemic renal failure [197]. BQ 123 also protects against the decline in renal function produced by arterial clamping [198, 199].

The immunosuppressant agents, cyclosporin A (cys A) and FK506 both have the potential to induce acute renal failure and endothelin has been proposed as the mediator. Cys A and FK506 have both been shown to stimulate ET-1 release from cultured kidney cells [200]. An endothelin antibody prevented the reduction in function induced by cys A in a rat kidney perfusion model [201]. In the same model, BQ 123 also prevented the detrimental renal effects of cys A [202].

The potential nephrotoxic effects of RCM have also been recognised. Again, endothelin may be a mediator. In a pacing-induced heart failure dog model, intravenous infusion of RCM produced significant elevation of plasma and urinary endothelin [203]. Similarly, RCM-induced elevation of plasma endothelin has been observed in the rat [204] and in man [205]. Furthermore, the ET receptor antagonist, CP170687, attenuated the fall in renal blood flow induced by RCM in the rat [206].

Other cardiovascular disorders

A number of other cardiovascular indications have been suggested, based mainly on the observation of raised endothelin levels and the known pharmacological effects of endothelin. Among the proposed indications are hypertension [207,208], pulmonary hypertension [209], atherosclerosis [210] congestive heart failure [211], shock [212] and Raynaud's disease [213].

NON-CARDIOVASCULAR DISEASE

Asthma

The bronchoconstrictor [39, 40], mitogenic [41] and extravasatory [42] actions of endothelin are consistent with a role in asthma. Increased

endothelin levels have been detected in the bronchiolar lavage fluid of asthmatic subjects, and these have returned towards normal after successful treatment of the condition with corticosteroids [214]. Immunohistochemistry has also revealed a marked increase in endothelin expression in airway epithelium from asthmatic patients compared with healthy subjects [215]. The ET_A antagonist BQ 123, by either intravenous or aerosol administration, has been shown to attenuate the late phase antigen-induced bronchoconstriction in the allergic sheep model [216].

Other diseases

Other non-cardiovascular disease states in which endothelin has tentatively been implicated, albeit on rather limited evidence, include some forms of cancer [217, 218], arthritis [219], liver cirrhosis [220], scleroderma [221], ulcerative colitis and Crohn's disease [222].

CONCLUSIONS

The profound pharmacological effects of endothelin, particularly on the cardiovascular system, are consistent with it playing an important pathological role in a number of disease states. Consequently, great efforts have been made to develop inhibitors of the action of endothelin in anticipation of such agents having considerable therapeutic utility. Of the two approaches taken, only limited advances have been made in the identification of inhibitors of the formation of endothelin by an endothelin converting enzyme, whereas both peptide and non-peptide antagonists of endothelin receptors have been identified. It remains to be seen whether the determination of clear clinical targets will allow these agents to be further developed into useful therapeutic agents.

ACKNOWLEDGEMENTS

The authors would like to thank Mrs S Stoggall and Miss S-J Slee for providing Figures 8.3–8.5 and Mr R James for valuable discussions.

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Progress in Medicinal Chemistry – Vol. 31, edited by G.P. Ellis and D.K. Luscombe

9 Potassium Channel Activators: Pharmacological Methods, Models, and Structure–Activity Relationships

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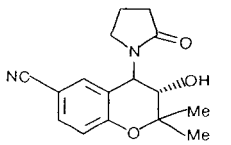
INTRODUCTION

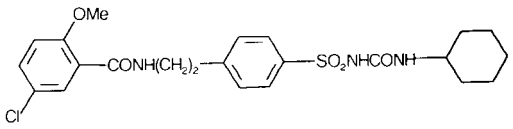
Potassium channel activators (KCAs), or openers, are a group of compounds that have aroused considerable pharmaceutical interest during the last decade as smooth muscle relaxants. Following the publication in 1986 [1] of this novel mechanism of action for cromakalim (1), virtually every major pharmaceutical company has initiated a programme to study the mechanism of action and structure–activity relationships (SAR) of this group of compounds, particularly benzopyrans related to cromakalim (1) and its active 3*S*,4*R* enantiomer, levcromakalim (2) [2].



(1) cromakalim (±)

(2) levcromakalim (3*S*, 4*R*)

An initial viewpoint that this mechanism of action would not produce effects other than those of simply closing voltage-operated calcium channels, and thus offer little advantage for KCAs over calcium antagonists at relaxing smooth muscle, was rapidly changed by studying the detailed pharmacology of the KCAs. A major assumption was that the mechanism of action of cromakalim (1), in particular, was entirely due to opening of smooth muscle membrane potassium (K^+) channels. The discovery [3] that insulin-releasing compounds, such as the sulphonylurea glibenclamide (3), inhibit the actions of the KCAs focused mechanistic studies on the adenosine triphosphate-regulated (closed by ATP) K^+ channel (K_{ATP}). However, evidence has accumulated that this mechanism may be too simple, and that the KCAs may possess additional mechanisms of action such as the



(3) glibenclamide

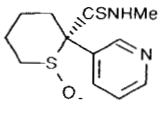
ability to affect intracellular calcium stores [4], and to open additional K^+ channels that are glibenclamide-sensitive yet ATP-insensitive [5]. It has also been reported that some KCAs may be able to open K^+ channels in one tissue and block K^+ channels in other tissues [6, 7], and that different KCAs open different K^+ channels [8].

At the same time, ideas on the potential therapeutic uses of KCAs have been modified as the understanding of their pharmacology has increased. The original indication was for the treatment of hypertension but this has been extended to include [3] angina, urinary incontinence, irritable bowel syndrome, epilepsy and bronchial asthma.

This review covers recent advances in studies of SAR of the various types of KCAs, reports on the compounds known to be in development, and highlights how structurally similar KCAs can have diverse pharmacological effects. The pharmacological methods and the various animal models used for the screening of KCAs are outlined, and differences between the compounds in these screens are noted. The second generation KCAs and prospects for future research are also discussed.

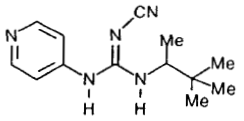
STRUCTURE–ACTIVITY RELATIONSHIPS OF KCAs

This topic has been reviewed both generally [9], and specifically for variation in the benzopyran nucleus [10]. SARs have also formed part of several comprehensive and useful reports and reviews [11–15], while full details of lead and development KCAs are regularly updated in a useful compilation [16] devoted to modulators of K^+ channels. Hence, this section will address recent advances, and those previously reported [9, 10], where further detail is now available. These are best presented in terms of the different series of KCAs based on the prototype molecules, cromakalim (1), RP 49356 (4), pinacidil (6), and nicorandil (7) that, together with the older compounds, minoxidil sulphate (8) and diazoxide (9), now recognized as KCAs, illustrate the widening range of structural types of this classification

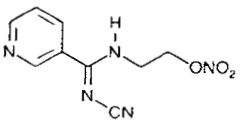


(4) RP 49356 (±)

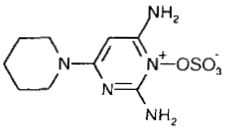
(5) aprikalim (1*R*, 2*R*)



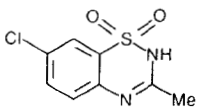
(6) pinacidil (±)



(7) nicorandil



(8) minoxidil sulphate



(9) diazoxide

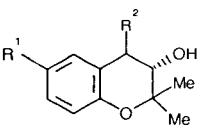
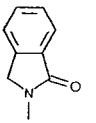
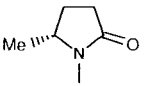
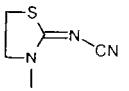
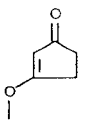
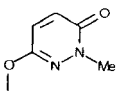
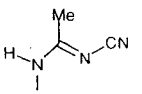
Figure 9.1. The range of KCA structural types

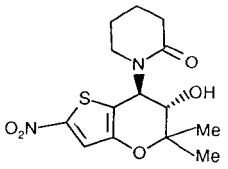
(*Figure 9.1*). An additional section is devoted to new structures that have recently been reported as KCAs.

BENZOPYRAN SERIES OF KCAs

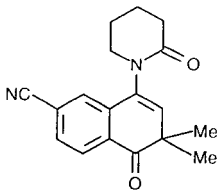
The benzopyran KCAs continue to attract more interest than the other structural types, as *Table 9.1*, which lists some of the earlier lead and development compounds (10)–(17) [9], confirms. The benzopyran nucleus has been replaced by a range of nuclei [9] to which can now be added thienopyrans, 1-tetralones, and benzoxazines. All possible combinations of thienopyran are described [17] with the progression of the antianginal 2-nitrothieno[3,2-*b*]pyran, RWJ 29009 (18), that was said to be an order of magnitude more potent than cromakalim (1) in reducing blood pressure in spontaneously hypertensive rats (SHR). Although certain analogues of the recently developed 1-tetralone series were reported to be airways-selective

Table 9.1. SOME LEAD BENZOPYRAN KCAs (10)–(17)

Compound No.	R^1	R^2 	*Trans stereochemistry C-3–C-4*	*Name/Code*
(10)	NC		$\Delta^{3,4}$ (chromene)	Bimakalim
(11)	NC		3*S*,4*R*	emakalim
(12)	CF_3O		3*S*,4*R*	celikalim
(13)	NC		3*R*,4*S*	S0121
(14)	NC		3*S*,4*R*	FR 119748
(15)	NC		3*S*,4*R*	SDZ PCO 400
(16)	NC		3*S*,4*R*	EMD 57283
(17)	NC		(±)	KP 294

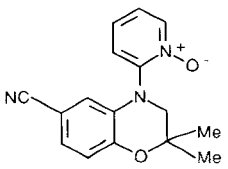


(18)

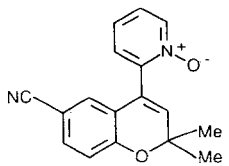


(19)

[18], no lead compound has been specifically identified. However, an example of the series, UR 8225 (19), was about as potent as levcromakalim (2) in relaxing the noradrenaline-induced contraction of rat isolated portal vein, and in inhibiting spontaneous contractions in guinea-pig isolated tracheal spirals (GPTS) [19]. This compound did not display any selectivity in these *in vitro* tests and any claim to selectivity within this series is anecdotal at present. The best compound of a series of 1,4-benzoxazine compounds, YM-934 (20), was said to be about tenfold more potent than cromakalim (1) in inhibiting spontaneous contractions in rat isolated portal vein [20]. The structural similarity of this compound to Ro 31-6930 (21) is quite striking, as is its similar degree of potency. Compound (21) was designed [21], and synthesized [22, 23], as a novel KCA with the key modification being the replacement of the C-4 lactam of (1) by the bioisosteric pyridine *N*-oxide group.



(20)

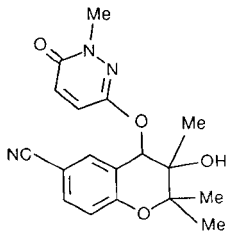


(21)

Both in the early publication [21] and in a more recent paper [24], the requirement for the gem dimethyl group in cromakalim (1) was addressed, and a similar conclusion to that noted previously [25] reached, that its presence was crucial to high potency. However, it is unclear whether this is because it confers conformational stability, or possibly less likely, because of a lipophilic binding interaction with the putative receptor. It is interesting to note that increasing the overall lipophilicity of KCA benzopyrans extends

the duration of their antihypertensive effect [26]. For example, celikalim (12) (see *Table 9.1*) is one of the more lipophilic and long acting KCAs. In addition, this property of extended duration of effect of the more lipophilic KCAs appears to be independent of their potency.

The insertion of an additional methyl group at the pyrrolidinone C(5′) position enhances the antihypertensive activity seen in compound (1), but the 5′*R*,3*R*,4*S* diastereomer, S 0121 (13) (see *Table 9.1*), is said to relax selectively the rat isolated ureter, and thus have a potential application in easing the passage of kidney stones [27]. Subsequently, a methyl group has been inserted in addition to the hydroxyl group at C-3 in the conventional cromakalim (1) series of KCAs [28]. This caused an attenuation of *in vitro* relaxant potency. In marked contrast, in the *O*-linked compounds such as (22), potency was enhanced. A likely explanation is the probable effect of this methyl group on the orientation of the C-4 substituent. A close to an orthogonal disposition [29] with respect to the benzopyran nucleus is thought to be required for optimal potency. In this case an orthogonal relationship is attained only in the *O*-linked series.

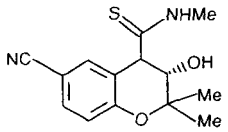


(22)

The role of the C-3 hydroxyl group has been investigated by comparing a series of enantiomeric chromans and *trans*-chromanols related to cromakalim (1) and Ro 31-6930 (21), for their relaxant effects in rat isolated portal vein [30]. In the cromakalim (1) series there was an approximate two-fold difference in potency between the enantiomeric chromans, but the difference was increased to about one hundred-fold in the pair of enantiomeric chromanols. Thus, it was shown that the absolute stereochemistry of the hydroxyl group has a considerably greater influence on potency than the absolute stereochemistry of the C-4 substituent in the cromakalim (1) molecule. This effect was less pronounced in the chromans and chromanols related to compound (21).

Variation in the C-4 substituent continues to be one of the most fruitful

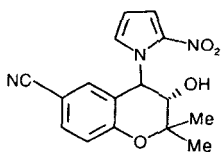
approaches to generation of benzopyran KCAs. Replacement of the pyrrolidinone group of cromakalim (1) by the methyl thiocarboxamide group, the ubiquitous feature of the aprikalim (5) (see *Figure 9.1*) series of KCAs, provided a compound (23) of almost a magnitude higher potency than both compounds (1) and (5), in its ability to inhibit spontaneous contractions in GPTS. Compound (23) also showed *in vivo* bronchodilator activity against histamine-induced effects in the guinea-pig [31]. Appropri-



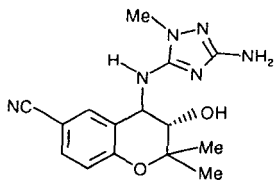
(23)

ately substituted pyrroles have produced useful non-carbonyl equivalents of pyrrolidinones [32], for example, the 2-nitro compound (24), where it is proposed that the substituted enamine moiety mimics the amide group, to produce a potent effect against spontaneous tone in GPTS.

Further details of studies of *N*-linked triazoles at the C-4 position have emerged [14] that indicate that the presence of the *N*-methyl group in compound (25) is pivotal in raising antihypertensive potency in SHR by an



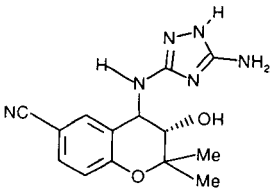
(24)



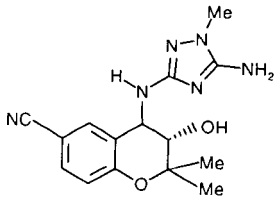
(25)

order of magnitude over the de-methyl compound (26), and three-fold over cromakalim (1). Compound (26) was shown by solution NMR to exist in the tautomeric form corresponding to that found in the isomeric *N*″-methyl analogue (27). It is notable that compounds (26) and (27) have similar potencies.

A publication [33] dealing with the interposition of the C-4 and C-3 groups has detailed the SARs associated with this interesting departure from the usual benzopyran KCA substituent pattern. Thus, while the *trans*

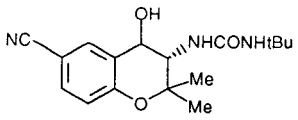


(26)

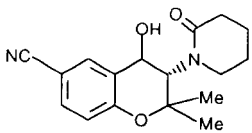


(27)

t-butyl urea analogue (28) had levels of antihypertensive potency equivalent to (1), the methyl urea counterpart was noticeably less potent, a reversal of the SAR found for C-4 substituted ureas [9]. Of even greater interest was the almost complete attenuation of activity in the piperidinone analogue (29).

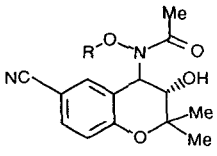


(28)

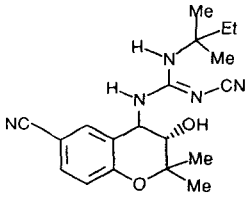


(29)

The search for benzopyran KCAs that offer differing cardiovascular profiles from, and selectivity for, other tissues over the lead antihypertensive drugs such as levcromakalim (2), seems to be achieving some success, with the appearance of so-called second generation compounds. Thus Y-27152 (30) (R = $PhCH_2$) exerts its antihypertensive effects via an active metabolite, Y-26763 (30) (R = H) that is formed by hepatic cytochrome P450 mediated debenzylation *in vivo* [34]. Y-27152 (30) (R = $PhCH_2$) induces a gradual decline in blood pressure in the conscious renal hypertensive dog (Goldblatt) model, and is accompanied by a reduced tachycardia, a potentially useful profile. Certain KCAs that possess little propensity to reduce blood pressure, for example, U-89232 (31), are reported to have potential for myocardial protection, as judged by their ability to reduce infarct size in the anaesthetized occluded coronary artery rabbit model [35]. The reduced antihypertensive effect of compound (31) is reflected in its poor ability to relax methoxamine-induced contractions in rat aorta [36].

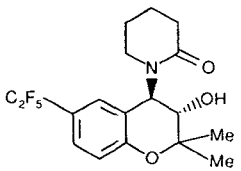


(30)

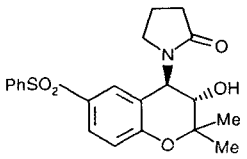


(31)

BRL 55834 (32) has been shown in *in vivo* models [37] to be about an order of magnitude more selective than levcromakalim (2) for the airways compared with blood vessels (see below for a more detailed discussion), a profile change that has potential in the treatment of asthma. Although the cardiovascular [38] and airways [39] activity of HOE 234, rimakalim, (33) have been defined, there is some speculation that this compound too, may possess selectivity for the airways.

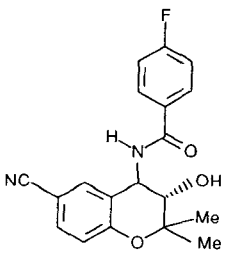


(32)



(33)

A potential advance in benzopyran SAR is reported [40] with a series of *trans*-C-4-substituted 4-fluorobenzoyl-amino compounds, such as (34), that



(34)

are useful as anticonvulsant agents. Further details, such as cardiovascular profiles, of these compounds are required before it can be decided whether selectivity for a CNS action has been achieved.

APRIKALIM SERIES OF KCAs

This series of KCAs, developed originally from a study of potential K^+/H^+ ATPase inhibitors resulted in the lead compound RP 49356 (4), and its biologically active 1*R*,2*R* enantiomer, aprikalim (5) (see *Figure 9.1*) [41]. The precursor sulphide of (4) was inactive *in vitro*, but careful observation of its effect in SHR identified a delayed onset in antihypertensive activity that led to the identification of the *trans* sulphoxide as its active metabolite. In contrast, the *cis* diastereomer was significantly less active.

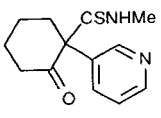
Initial SAR data on this series of compounds have been obtained [42], by observing the ability of analogues to inhibit 20 mM KCl induced contractions in rat isolated aorta. Thus replacement of the 3-pyridyl group of compound (4) by 2- or 4-pyridyl groups resulted in loss of potency. However, replacement by a 3-quinolyl substituent resulted in a ten-fold increase in potency. Although the use of other heteroaryl groups attenuated activity, certain substituted phenyl groups provided useful bioisosteric replacements. Thus, strong electron-withdrawing groups such as cyano and trifluoromethyl at the key meta position of the phenyl ring resulted in potent compounds. This is a situation not unlike that observed in both the cromakalim (1) and pinacidil (6) (see below) series where pyridyl and cyanophenyl groups are possible bioisosteres.

Only small *N*-alkyl groups can be tolerated on the thioamide group, optimal potency being associated with the methyl group with activity declining for larger substituents, an observation paralleled in the C-4 thioamide substituent in the benzopyran series [31] discussed above.

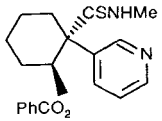
The acyclic ring incorporating the sulphoxide group can be replaced by other saturated heterocyclic rings, but activity is attenuated [42]. However its replacement by a cyclohexanone group, as in analogue (35), provided an *in vitro* potency, $IC_{90} = 0.4\ \mu M$, similar to that of compound (4) [43]. Moreover, replacement of the carbonyl group by a 2S-benzoate group [44] has furnished one of the most potent KCAs, RP 66471 (36), having an $IC_{90} = 0.3$ nM, but unfortunately lacking selectivity for either airways or the vasculature [45].

PINACIDIL SERIES OF KCAs

Replacement of the pyridyl group in this series has led to new KCA

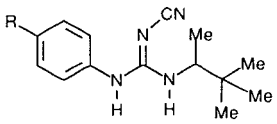


(35)



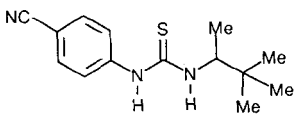
(36)

molecules [46], much as in the cromakalim (1) and aprikalim (5) series, described previously. Thus it was found that incorporation of the cyanophenyl group provided potent molecules such as BMS 182264 (37) (R = CN) that possessed an IC_{50} of 0.022 μM against methoxamine-induced contractions in rat aorta, but surprisingly, in contrast to the cromakalim (1) series, the nitro analogue (37) (R = NO_2) was about two orders of magnitude less potent as a relaxant of rat isolated aorta. This finding is apparently contrary to the concept of bioisosterism of a phenyl group bearing a strong electron withdrawing group, and a pyridyl group, in this series.



(37)

Although the original study on pinacidil (6) analogues (see *Figure 9.1*) [47] described thioureas as possessing fairly low antihypertensive activity, the thiourea containing the bioisosteric cyanophenyl group, BRL 49074 (38) is a potent relaxant of vascular smooth muscle. The unusual feature of this compound is its ability to function as a K^+ channel blocker in GPTS [7] (for further discussion, see below), and unlike pinacidil (6), the two enantiomers of compound (38) had a similar profile of activity. An explanation of the activity of thioureas, such as (38), nitroethenediamines, and cyanoguanidines of the pinacidil (6) series has been proposed on the basis of conformational similarities, as these groups are able to adopt similar low-energy staggered conformations that could correspond to the active conformation at the putative receptor [48]. The similarity of the activity profile of the enantiomers of (38) could also be explained by the reduced bulk of the thiocarbonyl group compared with the nitroethenyl and cyanoimino groups which are thought to make an important contribution to the enantioselectivity due to the 1,1,2-trimethylpropyl terminal, as the

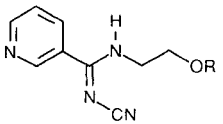


(38)

receptor is only able to accommodate the α-methyl and *t*-butyl groups in a certain conformation [48].

NICORANDIL SERIES OF KCAs

Nicorandil (7) (see *Figure 9.1*), the first compound shown to derive some of its pharmacological activity from K^+ channel activation [49], has been developed for the treatment of angina. Two additional compounds in this series, KRN2391 (39) (R = NO_2) [50], and FK336 (40) [51], appear to be in development for the same indication. The former is more than four orders of magnitude more potent than compound (7) in the vasopressin-treated rat model of angina.



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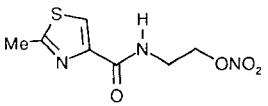


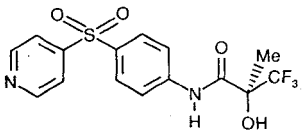
(40)

The nitrate moiety in KRN2391 (39) (R = NO_2) is of importance not only for its action in activating soluble guanylate cyclase, but also for the degree of potency of compounds (39) as KCAs. Thus, Ki3315 (39) (R = H) and Ki4032 (39) (R = COMe), are both KCAs but without the guanylate cyclase action, but both are less potent than compound (39) (R = NO_2) as KCAs [52]. Further comment is made below.

NOVEL KCA STRUCTURES

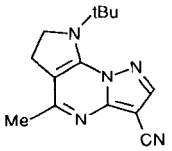
A potentially interesting advance for the treatment of urinary incontinence has been reported [53]. Although some of the analogues of a series of KCA substituted anilides lower blood pressure, others are stated to inhibit bladder contractions without cardiovascular effects. Thus, compound (41) increased the intercontraction interval of conscious rat bladder at a dose of

3 mg/kg, without alteration of blood pressure and heart rate up to 5 hours postdose.



(41)

LP-805 (42), a conventional vasodilator, owes some of its pharmacological action to K_{ATP} channel activation [54], whilst SCA40 (43) has been shown to open the large-conductance calcium-activated K^+ channel in GPTS and thus is of potential use in airways related disease [55]. Further discussion of the pharmacology of these compounds appears below. Compound (44) is a typical example from a class of compounds reported to open the large conductance (154 pS in symmetric 144 mM K^+) calcium-activated K^+ channel *in vitro* using cultured neuroblastoma cells, indicating a potential use in CNS disorders [56].



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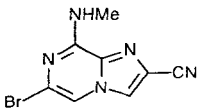

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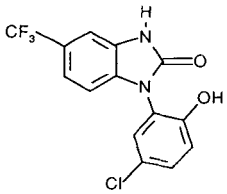


(44)

SCREENING ASSAYS

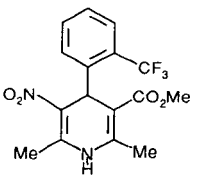
In developing screens for KCAs, the primary objective is to identify the general KCA mechanism of action, and to distinguish this from other mechanisms of action. This is because any programme of synthesis of potential KCAs could produce compounds which activate K^+ channels and also have (an) additional mechanism(s) of action as part of their inhibitory effect. Thus, it is important that a screening cascade can distinguish between

pure KCAs and multiaction compounds. Various approaches are required to resolve these problems, and these will be briefly reviewed.

HIGH AND LOW K^+-INDUCED CONTRACTIONS

It is one of the hallmarks of a compound acting solely through K^+ channel activation that it will relax low K^+-induced contractions in smooth muscle tissue, but not high K^+-induced tone. In contrast, a calcium channel antagonist will relax both high and low K^+-induced tone [3]. The problem with this approach arises when compounds which have additional mechanisms of action are discovered that will potently relax low K^+-induced tone, but will also induce a relaxation against high K^+-induced tone. A typical example is nicorandil (7), which activates guanylate cyclase to increase cGMP as well as opening K^+ channels. The use of low and high K^+ ion concentrations cannot adequately distinguish its mechanism from, for example, sodium nitroprusside, which is thought to act solely by increasing cGMP levels.

A further practical consideration of using low K^+ ion concentration to induce tone is that the contractions can be small and not well maintained. A possible solution to this problem is to enhance the tone by using the calcium agonist, Bay K 8644 (45), but this further complicates the assay [57]. A simpler alternative is to use a spasmogen-induced contraction that can be potently relaxed by cromakalim (1) (for example, noradrenaline-induced contraction in vascular tissue), and then compare the compound under examination against high K^+ ion concentration [58].

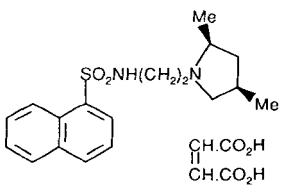


(45)

K^+ CHANNEL BLOCKERS

A subsequent approach is to use K^+ channel blockers which appear to have selectivity for the K^+ channels activated by the KCAs. The typical example is glibenclamide (3) which induces a rightward shift in the K^+ concentra-

tion–effect curve and reverses the relaxations under certain circumstances [59]. By comparing the effects of glibenclamide (3) on cromakalim-induced relaxations with the compound under test, the pA_2 and degree of antagonism can indicate whether the compound has an additional mechanism of action. In order to facilitate secondary evaluation, a K^+ channel blocker can thus be introduced into the primary screen. Thus, for instance, tone develops spontaneously in GPTS, and with appropriate mechanical manipulation, 1–2 g tension can develop in this tissue. This is relaxed by KCAs (but not calcium antagonists) with an intrinsic activity relative to a maximum relaxation induced with isoprenaline, of greater than 90%. Addition of (1 or 10 μM) glibenclamide (3) at the end of a full concentration-effect experiment, rather than inclusion from the start, does not reverse the relaxation. This may be due either to the competitive nature of the antagonism produced by glibenclamide (3) being unable to surmount the relaxation, or that glibenclamide (3) can induce relaxations of spontaneous tone in GPTS at concentrations greater than 3 μM. However, BRL 31660 (46), found to be a non-competitive K^+ channel blocker in random screening, was effective at reversing relaxations and was introduced into our screening cascade. This consisted of testing the putative KCAs over a standard concentration range of 10 nM–100 μM, and at the end of the experiment, either adding 10 μM compound (46), or if the compound under test had not fully relaxed the tissue, adding cromakalim (1) to induce a relaxation. Thus, if BRL 31660 reverses the relaxation, it suggests that the test compound is a KCA. If not, and the relaxation does not have the steep concentration–effect of a typical KCA, then it suggests that the test compound is not a KCA [31]. The problem with this approach is that, if a test compound can activate K^+ channels, but at the same, or a lower concentration, has an additional mechanism of action, then BRL 31660 will not reverse the relaxation, and the concentration-effect curve will be flatter than expected. If a test compound has a steep concentration–effect curve, and is not reversed by BRL 31660, it suggests that the compound has an additional mechanism of action at high concentrations, or it is activating a K^+ channel that is not susceptible to compound (46).



(46)

If the candidate compound does not relax the tissue, then addition of a standard, such as cromakalim (1), at the end of the experiment can serve two purposes. Firstly, if cromakalim (1) relaxes the tissue, it acts as a positive control, and indicates that the test compound is inactive. Secondly, if cromakalim (1) does not induce a good relaxation, it suggests that the test compound may be a K^+ channel blocker. BRL 31660 was found to block cromakalim-induced relaxations following this protocol, and as outlined above, has proved to be a very useful pharmacological tool.

EFFLUX EXPERIMENTS

A compound that opens K^+ channels should allow potassium to leave the cell down its concentration gradient. By loading tissues and/or cells with either $^{42/43}K^+$ or $^{86}Rb^+$ ions, this outward flux of ions can be measured [1]. In general, efflux experiments have been used to confirm that a test compound is a KCA. Unfortunately, this assay is time-consuming compared with the screens already mentioned, and is further complicated by the problems of the high specific activity of $^{42/43}K^+$ and $^{86}Rb^+$, and the relatively short half-lives (12.4 and 22.4 hours for $^{42}K^+$ and $^{43}K^+$ respectively, and 18.7 days for $^{86}Rb^+$) of these radioisotopes. Furthermore, the ability to induce K^+ efflux is not necessarily indicative of a K^+ channel-opening mechanism, as compounds such as noradrenaline can increase K^+ efflux by increasing intracellular calcium concentrations, and opening calcium-activated K^+ channels. Thus, the experiments outlined in the preceding two sections still need to be performed, to confirm fully the classification of a KCA. The advantage of efflux experiments is that they can identify compounds that activate K^+ channels and have an additional mechanism of action, provided the assay is sensitive enough. A method for using $^{86}Rb^+$ efflux as a rapid throughput assay using a 96-well microtitre plate has been reported [60]. This methodology can be automated and is ideal for cell cultures. As only low levels of radioactivity are required, this system may gain importance if routine cloning of K_{ATP} channels occurs, and their incorporation into cell lines allows natural product screening for novel chemical entities.

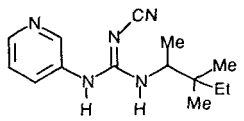
ELECTROPHYSIOLOGY

In the initial publications [1, 61, 62] on the mechanism of action of cromakalim (1), intracellular recording showed the development of a large membrane hyperpolarization. However, hyperpolarization *per se* is not necessarily due to an opening of K^+ channels, and supportive evidence from similar experiments to those outlined above is required. Intracellular

recording techniques have not been routinely used in screening cascades due to the technical expertise and the time required. Furthermore, these experiments provide little information on the class of K^+ channel being opened. The patch clamp technique has been widely used to investigate the properties of KCAs with recent publications [63–65] proposing the regulatory mechanisms involved in their effects on K_{ATP} channels. Again, this technique, although extremely useful for mechanistic studies, has not been extensively used in screening.

LIGAND BINDING EXPERIMENTS

Unlike many screening protocols where binding experiments are often the primary screen, in the KCA area very little progress has been made, due in the main to the lack of useful radioactive ligands and the lack of binding to membrane fractions or cultured cells. However, a pinacidil analogue, [^{3}H]-P1075 (47) that binds with high affinity to rat aortic tissue has been

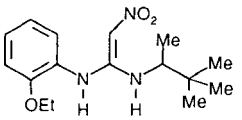


(47)

synthesized [66], but surprisingly, it did not bind to membrane fractions. Studies showed that with a diverse range of KCAs, all the compounds bound to a common site in rat aortic tissue, but surprisingly did not bind to membrane fractions. Interestingly, glibenclamide (3) was coupled in a negative allosteric manner to this KCA binding site. The study correlated the binding with functional activity and demonstrated stereoselectivity. In contrast to this report, a recent investigation using [^{3}H]-cromakalim (1) as the ligand in smooth muscle cells grown in culture, demonstrated the existence of a benzopyran binding site which was stereoselective, but, interestingly, the other diverse structural types of KCAs did not displace the ligand [67]. This suggests that the benzopyran KCAs may have different binding sites from the other series of KCAs, and further studies are required to resolve these inconsistencies. In support of the argument that the KCAs may bind at different sites, levcromakalim (2) has been reported [68] to inhibit binding of [^{125}I]-labelled endothelin-1 to rat cardiac membranes. However, benzopyran KCAs do not inhibit endothelin-induced contractions with the same potency as contractions due to other spasmogens [69].

This difference in potency ratios was not found with the aprikalim (5) or pinacidil (6) structural types. Thus, an explanation may be that the benzopyran KCAs bind at an endothelin-1 recognized site and an endothelin-1 independent site, and that the latter is influenced by aprikalim (5) and pinacidil (6).

Recently [70], high affinity binding with [^{3}H]-BAY X 9228 (48), a structural analogue of pinacidil (6), has been reported in a rat insulinoma (RINm5F) cell line. The IC_{50} values for inhibiting binding correlated with the ability of the KCAs at relaxing rat aorta. Interestingly, compound (48) differed in its potency in the two assays suggesting that receptor differences may exist between rat aorta and RINm5F cells. The number of glibenclamide (3) binding sites was 2.5 times higher than the number of compound (48) binding sites, which supports the suggestion that the sulphonylureas bind at a different, but very closely associated, binding site to the KCAs [66].



(48)

A major difficulty with these binding studies is the lack of effect on isolated membranes that does not permit the rapid screening normally associated with binding experiments. However, these experiments may lead to a greater understanding of the biochemical processes involved in the opening of K^+ channels by KCAs, and thus establish the conditions for the development of a screening assay that uses a membrane fraction.

IN VIVO MODELS

Cromakalim (1) [2] and RP 49356 (4) [41] were both discovered during *in vivo* screening using SHR, when very potent vasodilator effects were observed on administration of their precursor pyrrolidine and tetrahydrothiopyran compounds, respectively. Isolation of these active metabolites and their resolution led to levcromakalim (2) and aprikalim (5), respectively. SAR work for antihypertensive evaluation was generally performed in the SHR, but for evaluation as relaxants of airways tissue, *in vitro* models such as those outlined above were used initially, and further evaluation was only

carried out *in vivo* on lead compounds. The appropriate model for testing a potential KCA depends on the proposed clinical application, although the method of administration (intravenous or oral) may not always be the preferred route to be used clinically. Thus, for example, the inhaled route may be more appropriate for asthma therapy.

This section will briefly mention a few of the methodologies commonly used for the evaluation of antihypertensive and antiasthmatic compounds referred to in this review.

ANTIHYPERTENSIVE MODELS

The SHR is frequently used to study vasodilator compounds and has several advantages over other hypertensive models, although it is always possible that the decrease in blood pressure could be induced by a different mechanism from that found in man [71]. The SHR, a genetically bred animal which develops high blood pressure during normal development, is used to test potential antihypertensive KCA compounds. Since blood pressure can be measured indirectly by the tail-cuff method, this is a non-invasive, reliable, and robust screening method. The blood pressure changes can be monitored on a discontinuous basis for chronic dosing studies [72].

Direct measurement of blood pressure requires insertion of a cannula into an artery and generally a vein, respectively, to allow blood pressure and heart rate to be monitored, and also the administration of drugs. The animal is allowed to recover from anaesthesia, and the cardiovascular parameters are monitored in the conscious animal via the arterial cannula.

ANTIASTHMATIC MODELS

A recent review [73] describes the various animal models used to mimic the different aspects of this disease. For KCAs, the *in vivo* models employed have generally been bronchoconstrictor in nature, where test compounds have been examined for their ability to prevent or reverse an induced bronchoconstriction. This can be achieved in several ways, the simplest being the respiratory embarrassment model, where a guinea-pig is exposed to a concentration of a bronchoconstrictor that causes dyspnoea. The KCAs may be tested by the oral route for their ability to attenuate the onset of dyspnoea. Even in the control animals, in which dyspnoea leads to rapid collapse, removal of the animals from the chamber and away from the bronchoconstrictor agent, allows a full recovery [74]. Various other ways of inducing bronchoconstriction in conscious animals are available, one of the

most widely used being antigen-induced bronchoconstriction. Typically, the animals are sensitized initially by the intraperitoneal injection of 1 mg of ovalbumin and 0.2 mL of pertussis vaccine and then challenged with antigen (ovalbumin) by various routes, after a two week period. This challenge may not be severe enough to cause the animals to collapse, thus the time to a clearly defined phenomenon such as head jerk can be used.

These experiments are performed in conscious animals, and no effect on other parameters such as blood pressure and heart rate can be monitored unless the animals are anaesthetized. However, the use of anaesthetics can reduce the blood pressure and inhibit neurogenic reflexes, thus masking any changes in cardiovascular parameters. Nevertheless, by careful comparison with standard drugs, relative falls in blood pressure can be assessed between compounds.

The two main protocols used are the Könsett Rossler method and the respiratory dynamics technique [74]. Both methods involve anaesthetizing the animals and then administering compounds through a venous cannula and monitoring blood pressure and heart rate from an arterial cannula. The trachea is also cannulated for measurement of airflow and artificial respiration when necessary. In the Könsett Rossler model, since air is artificially pumped into the lungs, a neuromuscular blocking agent is administered to prevent spontaneous breathing. The volume of air which overflows is measured. If the animal bronchoconstricts (for instance, after administration of a compound like histamine), then less air is able to enter the lungs and so more air overflows. Thus, the volume of air that overflows reflects the degree of bronchoconstriction. If an anti-bronchoconstrictor agent is administered to the guinea-pig before the histamine challenge, then the increase in overflow is inhibited. This method gives no indication of which part of the bronchial tree is contributing to the bronchoconstriction or bronchodilation. By the insertion of another cannula into the oesophagus to measure pleural pressure, and computer analysis, the respiratory dynamics model is capable of acquiring these data. The parameters measured are airways resistance (considered to be the responses from the large airways) and dynamic lung compliance (responses from the small airways). During a respiratory cycle, volume and pressure changes occur. Measurements taken at particular stages of the cycle, can be used to calculate the two parameters.

This latter method has the advantage that neuromuscular blocking agents are not required since air is not being forcefully pumped into the lungs. This allows the spontaneous breathing of the animal to be used to study both bronchoconstrictors and bronchodilators, as well as allowing interactions of these compounds to be studied.

PHARMACOLOGICAL PROFILE

It has been proposed that KCAs, such as cromakalim (1), induce their relaxations by opening K_{ATP}, the K^+ channels closed by ATP, and hyperpolarising the cell membrane. This causes closure of voltage-operated calcium channels and may also affect intracellular calcium stores, the overall result being a relaxation of smooth muscle tone. Other reviews [3, 11, 75–79] provide more detailed explanations of the mechanisms involved in KCA-induced relaxations.

In this section, the effect of the standard KCAs on well documented events such as smooth muscle relaxation will only be covered briefly, the reader being referred to earlier, more comprehensive publications. However, the differences between the standard compounds and closely related analogues will be emphasized, where relatively small structural changes have produced marked functional differences. In addition, mechanistic studies which may explain these observations will be described, as these could lead to alternative screens in the future to identify the next generation of KCAs.

BENZOPYRAN SERIES

This group of compounds has been the most extensively studied with several analogues such as cromakalim (1) and levcromakalim (2) having been tested in man. The compounds relax smooth muscle preparations, showing little selectivity for certain different tissues, but in general, being more selective for vascular tissue. Thus, levcromakalim (2) has a selectivity ratio of 0.4 when comparing its relaxant effects in the guinea-pig portal vein (GPPV, KCl (30mM)-induced tone) with GPTS (spontaneous tone).

One of the few compounds in this series which has shown tissue selectivity is BRL 55834 (32) [37, 80, 81]. In particular, compound (32) can inhibit bronchoconstriction induced by various spasmogens, in different species (rats and guinea-pigs), while having little effect on blood pressure [74]. BRL 55834 (32) is an effective anti-bronchoconstrictor agent by the oral, intravenous, intraduodenal and inhaled routes of administration. In comparison, levcromakalim (2) had a similar potency on vascular parameters and was less potent on airways. Levcromakalim (2) also had a longer duration of action on the vasculature and a relatively short-lived effect on the airways. *In vitro*, BRL 55834 (32) demonstrates a relative selectivity for the airways in direct comparison with levcromakalim (2) [80]. Its selectivity ratio (as defined above) is >1.0, but this must be compared with the selectivity ratio of 0.4 found with levcromakalim (2), resulting in a

relative selectivity ratio between the two compounds of approximately 3.0. It is also interesting that the potency of BRL 55834 (32) in inhibiting the tone in GPTS induced by a range of different spasmogens, remains relatively constant. In contrast, the potency of levcromakalim (2) as a relaxant in airways is variable, for example, being less potent against histamine-induced, than spontaneous, tone. Thus, the relative selectivity ratio of BRL 55834 (32) compared with levcromakalim (2) varies between approximately three- to ten-old. This difference could explain the ability of BRL 55834 (32) to inhibit bronchoconstriction while having no effect on blood pressure.

Nevertheless, the reason why BRL 55834 (32) demonstrates this selectivity for the airways is not fully understood. Mechanistic studies using the patch clamp technique have suggested that unlike levcromakalim (2), compound (32) is able to activate at least two K^+ channels in bovine trachea [82]. Both levcromakalim (2) and BRL 55834 (32) modulate K_{ATP} channels (30 pS conductance) but the latter is also capable of activating a calcium-dependent, large conductance K^+ channel (243 pS conductance) that is known to be present in high density in airways smooth muscle. Whether this effect can explain the airways selectivity of BRL 55834 (32) is uncertain, especially as the effects of compound (32) are inhibited by glibenclamide (3) and BRL 31660 (46), suggesting an effect involving K_{ATP} channels. Unfortunately, no experiments have been performed using functional measurements to ascertain whether charybdotoxin, a blocker of calcium-dependent large conductance K^+ channels exerts a greater inhibitory effect on the relaxant actions of BRL 55834 (32) relative to levcromakalim (2). Certainly, in patch clamp experiments, the effects of compound (32) on the large conductance calcium activated K^+ channel were inhibited by charybdotoxin, supporting an effect on this channel.

A further significant difference between levcromakalim (2) and BRL 55834 (32) is the time-course of their relaxations. Whereas the relaxant effects of levcromakalim (2) on histamine-induced tone diminish over 26 minutes, the relaxant effects of BRL 55834 (32) are well maintained for over 50 minutes [80]. This may suggest that compound (32) has the ability to maintain the K^+ channels in an open state for a longer time, or that desensitization does not occur as rapidly with compound (32).

Rimakalim (33) has a similar potency and duration of action to BRL 55834 (32). The effects of rimakalim (33) have been compared with those of levcromakalim (2) on bronchoconstriction induced in guinea-pigs and on GPTS [39]. *In vitro*, rimakalim (33) was six to ten times more potent than levcromakalim (2) and had a similar potency to BRL 55834 (32) for inhibiting, for example, spontaneous tone [39]. This compound (33) was

also capable of inhibiting histaminergic tone for significantly longer periods than levcromakalim (2). However, rimakalim (33) differed significantly from levcromakalim (2) in that it was twenty-five times more potent as an inhibitor of a low (0.03 μM) carbachol-induced contraction. In contrast to other standard KCAs [79], rimakalim (33) induced concentration-dependent relaxations with a relatively high intrinsic activity against contractions induced by higher concentrations of carbachol (0.3 μM). Thus, relative to a maximal relaxation induced by isoprenaline (1 μM), rimakalim (33) had an intrinsic activity of 0.83, whereas levcromakalim (2) had an intrinsic activity of only 0.26 [83]. The reason for this is unclear, but suggests that rimakalim (33) may have an additional action at muscarinic receptors, thus preventing the agonist action of carbachol. Alternatively, there could be a more fundamental difference, such as opening a different K^+ channel which has a greater effect on the intracellular calcium stores.

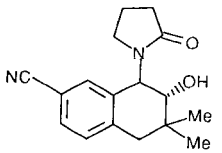
The cardiovascular effects of rimakalim (33) have been demonstrated in rats, dogs and monkeys [39]. Interestingly, this compound (33) is more potent than cromakalim (1) by the intravenous route, but by the oral route it is equipotent, or less potent than cromakalim (1). Comparison of its effects in conscious, orally treated rats with its actions in anaesthetized, intraduodenally-treated rats showed a delayed onset by the intraduodenal route, in contrast to cromakalim (1). One explanation could be that the compound is rapidly absorbed from the stomach but is only slowly absorbed from the rest of the small intestine. However, with the known effect of rimakalim (33) on muscarinic responses in the airways, and the important role cholinergic responses play in the gastrointestinal tract, an alternative hypothesis is that unlike other KCAs, rimakalim (33) will decrease gastric motility due to its anticholinergic effect. However, there has been very little mechanistic data published on rimakalim (33), and further studies are required to explain the differences between this compound and other KCAs. It is known that its effects are blocked by glibenclamide (3), but other suggestions as to the mode of action of this compound have generally been speculative.

Ro 31-6930 (21) is a more potent KCA than levcromakalim (2) and demonstrates no selectivity for a particular smooth muscle preparation. However, in a direct comparison of its effects on antigen-induced bronchoconstriction in guinea-pigs [84], Ro 31-6930 (21) was capable of inhibiting tone whereas levcromakalim (2) had no significant effect. This is in contrast to other data reported for these compounds, and the authors provided no explanation for this anomaly. A further difference in the profile between these compounds has been reported in dogs, where Ro 31-6930 (21) had no effect on renal vascular resistance, whereas cromakalim (2)

significantly reduced this parameter [85]. The antihypertensive effects of Ro 31-6930 (21) were reported to be longer acting than those of cromakalim (1) in addition to the compound being ten times more potent. Other interesting findings reported for Ro 31-6930 (21) are its inhibitory effects on mucus secretions from ferret submucosal glands [86]. This is probably due to an action on K^+ channels, as electrophysiological studies using ovine cultured submucosal cells identified a population of K^+ channels sensitive to this compound (21) [87]. Whether other KCAs have this action remains to be established, but clinically this mechanism could be important in conditions where excess mucus production or a decrease in mucus production could be beneficial, such as in the treatment of bronchitis. Other potent KCAs are known, such as bimakalim (10) (see *Table 9.1*), but appear to have very similar profiles to those of the KCAs as a class.

However, one compound which has highlighted the need for extensive profiling across several tissues is SDZ PCO 400 (15) (see *Table 9.1*). This compound relaxes smooth muscle tone in vascular and airways tissue and appears to be a typical KCA. However, in pancreatic ß-cells, unlike cromakalim (1) and other KCAs, compound (15) inhibits K_{ATP} [6]. Thus, SDZ PCO 400 (15) might increase insulin release in a similar manner to the sulphonylureas.

Within the benzopyran series, analogues that have relative selectivity for the vasculature compared with the airways have been relatively easy to discover, for example the tetrahydronaphthalene (49) which has an IC_{50} of 4.5 μM against K^+ elevated tone in GPPV, but does not attain an IC_{50} against spontaneous tone in GPTS. This compound (49) possesses antihypertensive activity *in vivo* in the SHR [88].



(49)

Recently, U-89,232 (31) has been reported to lack activity at K^+ channels in vascular smooth muscle, but opens K^+ channels in cardiac tissue and is more potent than cromakalim (1) [35], a contrasting profile to the standard KCAs. Both cromakalim (1) and U-89,232 (31) shorten the cardiac action potential which could be envisaged to be proarrthymic, but this may be counterbalanced by the inhibition of calcium entry producing an anti-

arrthymic effect, and it is this effect that predominates in some animal models. However, other studies have reported deleterious effects when using pinacidil (6) in other animal models [89], and the balance of proarrhythmic and antiarrhythmic effects could be important.

APRIKALIM SERIES

As outlined in the SAR section, this series has produced some of the most potent KCAs. However, unlike the so-called second generation of KCAs from the benzopyran series, such as BRL 55834 (32) and rimakalim (33), RP 66471 (36) does not possess the ability to inhibit various spasmogens in GPTS with a similar potency. Thus, against spontaneous tone in GPTS, compound (36) has an IC_{50} of 0.0067 μM, but against 5 μM histamine-induced tone the IC_{50} was 0.029 μM [80], whilst against low concentration K^+-induced tone in rat aorta, the IC_{90} was 0.0003 μM [44]. This profile of varying potency is similar to that found with levcromakalim (2) [79]. *In vivo*, when administered by the inhaled route, both compounds (36) and (2) had similar potencies against histamine-induced bronchoconstriction. The reason for these variable effects against different spasmogens is as yet unknown. It has been suggested that the prolonged inhibition of tone induced by BRL 55834 (32) and rimakalim (33) may be an indication of their ability to inhibit a range of spasmogens, but unfortunately this does not hold true as RP 66471 (36) also induces a prolonged inhibition of histamine-induced tone in GPTS [79].

PINACIDIL SERIES

Pinacidil (6) itself exhibits no selectivity for a particular smooth muscle type and at high concentrations has a mechanism of action that is independent of K^+ channel opening [3]. Thus, caution must be exercised when studying SAR relationships as this KCA-independent mechanism can be enhanced.

Modification of the pinacidil (6) structure has resulted in BRL 49074 (38), that pharmacologically may be considered one of the most mechanistically interesting compounds published to date. Thus, BRL 49074 (38) relaxes GPPV with an IC_{50} of 1.72 μM, and stimulates K^+ efflux, both effects being blocked by glibenclamide (3), a typical profile of a standard KCA [7]. But, in marked contrast, BRL 49074 (38) had a low intrinsic activity of 0.11 relative to a maximum relaxation of 1.0 in GPTS, and blocked the effects of cromakalim (1) as a relaxant and K^+ efflux enhancer. Its effect on efflux was interesting since in GPPV it induced a significant increase in $^{42/43}K^+$ efflux, but to a level considerably less than that induced by compound (1). This

may be because BRL 49074 (38) is a partial agonist, although no evidence has emerged from its relaxant effects on GPTS and GPPV. The blocking effect of BRL 49074 (38) was not confined to benzopyran KCAs, as the same effect was observed for compounds of the aprikalim (5) and pinacidil (6) series. Interestingly, its blocking action against cromakalim (1) was non-competitive, that is, loss of the maximum effect with increasing concentration of BRL 49074 (38), whereas against pinacidil (6) the inhibition was competitive in nature [83]. This suggests that BRL 49074 (38) is competing directly with pinacidil (6) for its binding site, whereas it indirectly blocks the effects of benzopyran KCAs. An alternative explanation is that at the higher concentration of pinacidil (6) required to overcome the blocking effects of BRL 49074 (38), the KCA-independent mechanism of action of pinacidil (6) assumes a greater importance.

BRL 49074 (38) is a racemate, and surprisingly both its enantiomers demonstrated similar profiles as relaxants of GPPV and as blockers of levcromakalim-induced relaxations in GPTS.

NICORANDIL SERIES

Nicorandil (7) was the first compound shown to activate K^+ channels, but because of its ability to stimulate soluble guanylate cyclase many of its effects can be explained by the compound's ability to increase cyclic guanosine monophosphate (cGMP). Second generation compounds such as KRN2391 (39) ($R = NO_2$) are up to thirty times more potent but have similar effects, the K^+ channel and soluble guanylate cyclase activations occurring at similar concentrations. The importance of the nitrate moiety for activation of soluble guanylate cyclase has been demonstrated by comparison of compound (39) ($R = NO_2$) with its two non-nitrate derivatives Ki 3315 (39) (R = H) and Ki 4032 (39) (R = COMe) [52]. Both these compounds were less potent than KRN2391 (39) ($R = NO_2$) at relaxing tone in rabbit aorta [52, 90] or canine coronary artery [52, 90] and the authors inferred that the nitrate moiety was important for both KCA activity and activation of soluble guanylate cyclase.

The role that these two mechanisms can play in a particular species or tissue can vary considerably. Hence in rat aorta, glibenclamide (3) non-competitively inhibits the actions of KRN2391 (39) ($R = NO_2$), and it was suggested that the opening of K^+ channels plays a considerable part in the relaxant effect of KRN2391 (39) ($R = NO_2$) [52, 90]. However, in rabbit aorta, glibenclamide (3) induced only a small rightward shift in the dose-response curve and the investigators [52, 90] concluded that opening of K^+ channels and activation of soluble guanylate cyclase both play a

significant role in the relaxant effects of KRN2391 (39) ($R = NO_2$). Certainly, in the presence of methylene blue, an inhibitor of soluble guanylate cyclase, glibenclamide (3) induced a marked rightward shift in the concentration-effect curve for compound (39) ($R = NO_2$).

NOVEL STRUCTURES

LP-805 (42) has been shown to open K^+ channels and to induce release of endothelium-derived relaxing factor, which is nitric oxide (NO) or a closely related substance, from rat aorta [54, 91, 92]. Similar effects have been observed in rabbit coronary artery, but direct comparison of its effects with acetylcholine, which releases endothelium-derived hyperpolarizing factor (EDHF), suggests that compound (42) activates a different K^+ channel. Thus, LP-805 (42) induces the release of NO which activates guanylate cyclase and also activates a glibenclamide-sensitive K^+ channel. It does not, however, induce the release of EDHF, and LP-805 (42) is capable of relaxing de-endothelized tissues although it is less potent [91]. The effects of LP-805 (42) were not blocked by charbydotoxin, and the hyperpolarization and characteristics of the relaxation induced in de-endothelised tissues were similar to those induced by cromakalim (1) or pinacidil (6) [91].

In contrast, SCA40 (43) has recently been reported to open charybdotoxin-sensitive K^+ channels in GPTS, although it also increases cyclic adenosine monophosphate (cAMP) levels at high concentrations suggesting that it may possess some phosphodiesterase inhibiting activity [49]. Interestingly, unlike the standard KCAs, SCA40 (43) was capable of relaxing carbachol-induced tone, while charybdotoxin inhibited its relaxant effects in a competitive manner with no significant effect on its maximum response. This would suggest that charybdotoxin and SCA40 (43) are competing for the same site on the calcium-activated K^+ channel. As airway smooth muscle cells have a high density of these channels, this compound (43) may be particularly suitable as a bronchodilator for the treatment of asthma. However, these channels are very widespread and the selectivity of SCA40 (43) for particular subtypes of the calcium-activated K^+ channels needs to be ascertained.

MECHANISM OF ACTION STUDIES

Although evidence for K^+ channel activation explaining the mechanism of action of the KCAs was described in early publications [1, 61, 62], and was soon followed by the discovery of the blocking effects of glibenclamide (3),

thereby suggesting an interaction with K_{ATP} [3], the direct evidence for an effect on these channels using electrophysiological techniques in smooth muscle is minimal. Indeed, several workers have suggested that the calcium activated K^+ channel is responsible for the effects of the KCAs [93–95]. Recently, however, evidence has started to accumulate, using whole-cell membrane K^+ currents [63–65] and patch clamp techniques [82], that the KCAs activate a small conductance (10-30 pS, depending on the conditions) ATP- and glibenclamide-sensitive K^+ channel. In particular, it has been proposed [65] that the KCAs open K^+ channels by competing with ATP for its binding site. Thus, by preventing ATP binding, the KCAs stop the normal inhibitory effect of ATP and the probability of channel opening is increased. In addition, it has been suggested that as the K_{ATP} channel has to be phosphorylated for it to function, that is, to open, the KCAs also interfere with this phosphorylation process and thus facilitate rundown of the channels. Consequently, the effects observed with the KCAs are due to a balance between their ability in displacing ATP to allow channel opening, and their influence on the phosphorylation site that results in channel inactivity. Certainly, it has been shown [65] that aprikalim (5) and P1060 (47) have different time-courses to their maximum effects. This hypothesis could explain the different time-course of effects seen with compounds such as levcromakalim (2), BRL 55834 (32), and rimakalim (33), where the latter two compounds have a delayed onset of action, at threshold concentrations, but maintain their inhibitory effects for far longer than levcromakalim (2) [79]. If this is indeed the case, then the reason why a lesser effect on the phosphorylation site delays the time to maximum effect and maintains the channel in its open state needs addressing. However, this suggests that the ATP inhibitory binding site and the phosphorylation site are closely associated.

If this hypothesis is true, then this could lead to a new screen for seeking the next generation of KCAs that exert their effects on the ATP binding site but without influencing the phosphorylation site. This could in theory, result in a maintained channel opening on an indefinite basis while the compounds are present.

Whether this can explain the mechanism of the selectivity seen with BRL 55834 (32) and rimakalim (33) remains to be proved. Certainly, the studies which have been reported using smooth muscle cells from the portal vein need to be extended to airways smooth muscle. This is particularly important for BRL 55834 (32) which has been reported to activate two K^+ channels in bovine airways smooth muscle and shows selectivity for the airways relative to levcromakalim (2). Similarly, it will be interesting to ascertain whether the selectivity for the vasculature found with compounds

such as BRL 49074 (38) and the tetrahydronaphthalene (49) can be explained solely on the basis of their differential effects on the two proposed sites, or if another K^+ channel is involved. It could be hypothesized that in the case of compound (38), the channel present in the GPTS is more susceptible to the dephosphorylation induced by this compound, and thus it blocks the effects of cromakalim (1) by simply inactivating the K^+ channel. The reason why KCAs can be selective for the vasculature with little effect on the airways requires further study, but an influence on a different K^+ channel cannot be ruled out. For example, in GPTS, extracellular rubidium has the effect of blocking the cromakalim-stimulated $^{42/43}K^+$ and $^{86}Rb^+$ efflux, but does not block cromakalim-stimulated $^{86}Rb^+$ uptake, suggesting that two populations of K^+ channels can be differentiated [96]. A study is also required to determine whether the KCAs exert their effect on the ATP binding site, as the binding studies with [^{3}H]-P1060 (47) [66] have suggested that allosteric interactions with compounds such as glibenclamide (3) can occur.

The binding studies [66] also highlighted that minoxidil sulphate (8), in contrast to the other KCAs, showed no correlation between its ability to displace [^{3}H]-P1060 (47) binding and its functional effects, supporting the suggestion that this compound opens a different K^+ channel.

METABOLISM STUDIES

A limited amount of work has been published on this topic. For cromakalim (1), a h.p.l.c. method has been developed [97] to detect unlabelled (1) and its major metabolites in human urine, as renal clearance is the principal excretory route. The metabolic fate of levcromakalim (2) has been determined [98], and the resulting metabolites have been identified in human urine using ^{14}C-levcromakalim (see *Figure 9.2*). The compound was extensively absorbed, and in human plasma levcromakalim (2) accounted for the majority of drug-related material, while no evidence was found for any *in vivo* interconversion of levcromakalim (2) to the 3*R*,4*S*-enantiomer [98].

The vascular effects of the metabolites of levcromakalim (2) have been assessed [99]. Of the metabolites depicted in *Figure 9.2*, the only one to exhibit relaxant activity against noradrenaline-induced spasm in rabbit isolated mesenteric artery, or spontaneous tone in GPTS, was the 3′*R*-hydroxy-pyrrolidinone (50). However, metabolite (50) was ten-fold less potent than levcromakalim (2), and because of its low occurrence, does not

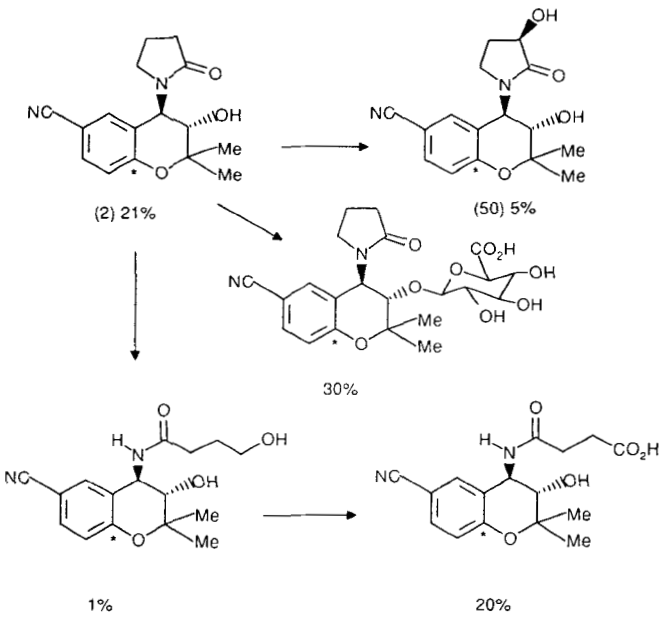


*Figure 9.2. Metabolic fate of ^{14}C-levcromakalim (2) deduced by analysis of human urine (0–144 h). *Denotes position of ^{14}C-radiolabel. % figures relate to mean radiometabolite pattern during the 0–144 h. [98].*

make a significant contribution to the biological activity of levcromakalim (2).

Pinacidil (6), like cromakalim (1), is mainly eliminated by renal excretion of a number of metabolites [100]. The principal metabolite is the pyridine *N*-oxide which is formed by hepatic cytochrome P450 mediated oxidation. The *N*-oxide possesses about a quarter of the vasodilator activity of pinacidil (6), but as renal clearance of this *N*-oxide is rapid, it does not constitute a problem in patients with normal kidney function.

CONCLUSIONS AND FUTURE PROSPECTS

There is little doubt that the investigation of the class of KCAs for the amelioration of a variety of disease states is gathering pace, and that selectivity for different tissues and organs is being sought by three approaches.

In the first, the known series of KCAs continues to be modified, so that compounds, such as **BRL** 55834 (32), have emerged that have a relative selectivity for the airways over the vasculature, possibly by the modulation of K^+ channels that are not opened by the more conventional members of the class. In the second, novel structures such as the benzimidazolone (44) that open different channels in different tissues are reported. In the third approach, compounds such as LP-805 (42) and SCA40 (43) have been discovered that have additional pharmacological actions, that together with K^+ channel modulation, are potentially advantageous for the treatment of certain disease states.

In pursuit of these goals the pharmacological screening and mechanistic studies are continually being refined. The recent reports of the cloning of an ATP sensitive K^+ channel from rat kidney [101], and the inward rectifier from a mouse macrophage line [102], suggest the advent of a new family of K^+ channel clones [103]. The availability of such channel clones, preferably human, will undoubtedly revolutionize the screening of potential new KCAs, and should lead to the discovery of more selective compounds.

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