THE DESIGN AND EVALUATION OF NOVEL

POLYAMINE-CYTOTOXIC CONJUGATES

AS ANTITUMOUR AGENTS

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ABSTRACT

A polyamine uptake system for the transport of
polyamines into tumours and rapidly proliferating cells
has been described. In this study, the effect of
conjugating cytotoxics to polyamine carriers on their
delivery and cytotoxicity to tumour cells *in vitro* and *in
vivo* was investigated.

In Ehrlich Ascites tumour cells, a number of
nitroimidazole-polyamine conjugates had a high affinity
for the uptake system but were not toxic. A
chlorambucil-spermidine conjugate was found to have a
high affinity for the uptake system and 30-fold greater
toxicity than chlorambucil to ADJ/PC6 plasmacytoma cells
in vitro. Polyamine depletion further enhanced the
toxicity of the conjugate. A 10,000-fold greater
reactivity with naked DNA compared to chlorambucil was
also seen. *In vivo*, however, chlorambucil-spermidine was
only two fold more active than chlorambucil.

A novel HPLC method for measuring chlorambucil
and chlorambucil-spermidine in biological samples showed
that the poor *in vivo* activity was not due to cleavage of
the conjugate. In ADJ/PC6 cells *in vitro* and all tissues
studied *in vivo*, there was enhanced uptake of
chlorambucil-spermidine compared to chlorambucil. There
was no apparent selective uptake of chlorambucil-
spermidine into tumour tissue, even when pharmacokinetics
and polyamine depletion were utilized in an attempt to
increase the delivery to tissues with a polyamine uptake
system. The role of the polyamine uptake system in the
uptake of the chlorambucil-spermidine conjugate was
unclear. In addition, unchanged chlorambucil-spermidine
could still be detected in tissues several days after
administration, suggesting that it may become bound
intracellularly at sites distant from its DNA target.

2

In four human ovarian carcinoma cell lines, the *in vitro* toxicity of chlorambucil-spermidine was greater than chlorambucil. Toxicity, however, did not appear to be related to the affinity of the conjugate for the polyamine uptake system or the ability of the different cells to accumulate polyamines.

In summary, conjugation of chlorambucil to spermidine increased its delivery and toxicity to tumour cells both *in vitro* and *in vivo*. Conjugation of a polyamine to a cytotoxic clearly increased its affinity for DNA but the role of the polyamine uptake system in the delivery of the conjugate was unclear. Competition for DNA binding by endogenous polyamines and intracellular binding of chlorambucil-spermidine at sites distant from the DNA may have contributed to the poorer than expected *in vivo* activity.

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CONTENTS

Chapter 1 - Introduction

Chapter 2 - Materials and Methods

2.2 Methods

CHAPTER 3 - *In vitro* and *in vivo* activity of a variety of polyamine-conjugates and analogues (including nitroimidazole-polyamine conjugates) to Ehrlich ascites tumour cells.

3.2 Results
3.2.1 Characterisation of spermidine uptake in Ehrlich
      ascites tumour cells: the effect of temperature

Chapter 8 - Final discussion

List of figures

List of tables

Abbreviations

AdoMetDC-		*S*-adenosylmethionine decarboxylase
AMP	-	adenosine monophosphate
ATP	-	adenosine triphosphate
BCNU	-	1,3-bis(2-chloroethyl)-1-nitrosourea
C-SAT	-	acetyl CoA : spermidine-spermine N^1 acetyl-transferase
CLB-SPD	-	chlorambucil-spermidine conjugate
DeC-PAAM-		dechlorophenylacetic acid mustard
DHS	-	donor horse serum
DFMO	-	α-difluoromethylornithine
DMEM	-	Dulbecco's Modification of Eagle's Medium
DNA	-	deoxyribonucleic acid
DMSO	-	dimethyl sulphoxide
EATC	-	Ehrlich ascites tumour cells
EDTA	-	ethylenediaminetetraacetic acid
FCS	-	foetal calf serum
GSH	-	glutathione (reduced)
GSH	-	glutathione-*S*-transferase
i.p.	-	intraperitoneal
i.v.	-	intravenous
MGBG	-	methylglyoxal-bis(guanylhydrazone)
ODC	-	ornithine decarboxylase
PAAM	-	phenylacetic acid mustard
PBS	-	phosphate buffered saline
PCA	-	perchloric acid
PUT	-	putrescine
RNA	-	ribonucleic acid
SD	-	standard deviation
s.c.	-	subcutaneous
s.e.m.	-	standard error of the mean
SPD	-	spermidine
SPN	-	spermine
TCA	-	trichloroacetic acid

CHAPTER 1 - Introduction

1.1 Introduction

Many tumours possess an energy dependent uptake system for the accumulation of polyamines and structurally related compounds. It may be possible to increase the delivery of cytotoxics to tumours via this uptake system by conjugating them to polyamine carriers. In this study, the potential use of polyamine-cytotoxic conjugates as antitumour agents was investigated. The importance of the polyamine uptake system for the delivery and toxicity of a chlorambucil-spermidine conjugate was also considered.

An overview of the structure, functions and metabolism of the polyamines is given together with a detailed examination of the polyamine uptake system. In addition, the exploitation of polyamines and polyamine uptake in chemotherapy is discussed. The mechanisms of alkylating agent activity and previous attempts at tumour targeting are also described.

1.2 Polyamines: structure and functions

Polyamines are ubiquitous low molecular weight organic cations which are required for cell growth and differentiation. The structures of the naturally occuring di-, tri- and tetramines, which are commonly designated "polyamines", are shown in fig 1.1.

At physiological pH the amino groups of the polyamines are protonated and from a structural point of view they are flexible molecules with positive charges distributed along the aliphatic carbon chain (1,2). The polycationic character is most pronounced for spermine because of its four positive charges (3). The polyamines form ion pairs with negatively charged (acidic) molecules and their affinity for acidic cellular constituents is

Fig 1.1 Structural formulae of the natural polyamines and of some drugs which share the polyamine uptake system

Putrescine

Spermidine

Spermine

MGBG

Paraquat
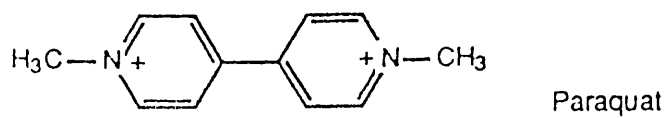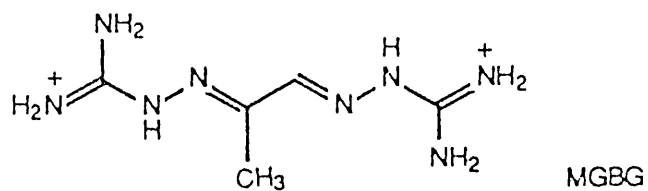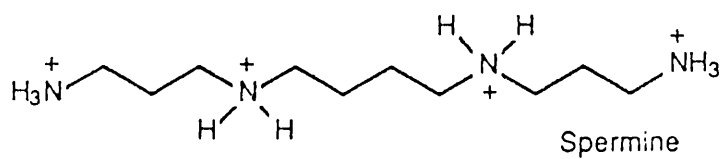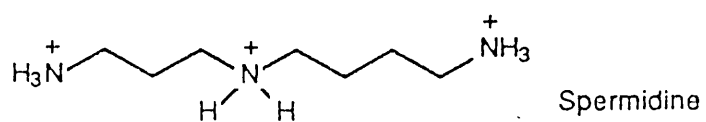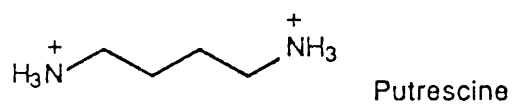
much greater than the cations K^+, Na^+, Mg^{2+} and Ca^{2+} which are point charges. It is the electrostatic interaction with DNA, RNA, proteins and negatively charged membrane constituents which is the basis for the majority of polyamine functions (2,4,5,6, 7).

The functions of polyamines are diverse and about 800 research reports have been published describing various effects exerted by polyamines in experimental systems (8). Many of their roles have been elucidated by studying the effect of inhibition of polyamine biosynthesis in cells which results in a depletion of polyamine levels.

In eukaryotes, spermine is thought to have a nuclear function since prokaryotes lack spermine but have putrescine and spermidine (9). Polyamine depletion results in inhibition of DNA synthesis and a subsequent reduction in the rate of cell growth (10,11,12). Polyamine starvation also causes major chromosome aberations in a polyamine dependent Chinese hamster ovary (CHO) cell line (13) and the disappearance of actin filaments and microtubules (14). Spermidine and spermine can bind to DNA through the interactions of their positively charged amino groups, with the tetramethylene portion of the polyamines bridging the minor groove and the trimethylene portion bridging adjacent phosphate groups (9). Therefore a role for polyamines in DNA stabilization is implicated (5).

Polyamines are involved in protein synthesis at the translational level where they may stabilize the protein synthesizing ribosomal complex (15). Polyamine depletion results in an inhibition of protein synthesis, which indirectly causes an inhibition of mitochondrial respiration. Polyamines may also regulate protein synthesis at the transcriptional level by their effects on chromatin associated nuclear protein kinases (16). Spermidine is also important for the stabilization of tRNA tertiary structure and rRNA subunits (3).

In addition to their electrostatic interactions, polyamines also form covalent bonds with glutamine residues of amino acids and proteins (17,18). Covalently bound polyamines, however, represent only a small proportion of the total polyamines in vertebrate tissues.

The relative proportions of polyamines in individual cells varies between organisms and tissues. In general, putrescine levels are lower than spermidine and spermine (2). Rapidly proliferating cells and tissues (embryos, tumours, small intestine, etc.) usually have higher putrescine and spermidine concentrations than the corresponding non-growing tissues (2). In some cases the ratio of spermidine to spermine may be indicative of the biosynthetic activity of the tissue, with a high spermidine:spermine ratio considered typical of a tissue undergoing hyperplasia (19,3).

Much interest has been focused on the relationship of polyamines with the growth status and proliferation of cells. Putrescine, spermidine and spermine levels increase progressively as cells traverse the cell cycle from G_1 to mitosis (19,20,21). The spermidine content of cells also shows a direct linear relationship with the specific cell growth rate, showing that spermidine accumulation is an event primarily associated with the process of cell replication (20). Studies by Goldstein et al. also indicate specific requirements for putrescine just before DNA synthesis and later during mitosis (22).

There is evidence that polyamines play a role in the differentiation of mammalian cells. Enhanced putrescine and spermidine levels are produced in response to agents inducing terminal differentiation in human HL-60 promyelocytic cells (23). In addition, embryonic development (24) and insulin induced differentiation of L6 myoblast cells (25) are inhibited if polyamine synthesis is prevented.

As a summary, due to their interactions with

nucleic acids, polyamines can affect virtually all processes in which nucleic acids are involved including replication, transcription and translation of genetic information (3). These compounds are therefore indispensible for cell growth irrespective of their exact modes of action.

In addition to their physiological properties, polyamines also have a number of pharmacological effects. Spermidine and spermine both demonstrate lethal CNS toxicity in mice (LD_{50}= 2.4 and 0.9 mmol/kg respectively) (2) which, together with direct measurements, suggests that they are capable of crossing the blood brain barrier to some extent (26,27).

The polyamines also have free radical scavenging properties which are thought to be responsible for their inhibition of lipid peroxidation and ulcer formation (28). Polyamines may also play a role in the spontaneous motor activity of the gastrointestinal tract (2).

The effects of polyamines on membrane functions have been reviewed (29). One of the major functional roles of polyamines at membrane sites is presumed to be influencing ion movement (2). Polyamines are also thought to play a role in calcium homeostasis(2). In addition, evidence suggests that the polyamines spermidine and spermine may be mediators of hormonal stimulation (3,30,31,32).

1.3 Polyamine biosynthesis and catabolism

1.3.1 Introduction

The biochemistry of the polyamines has been studied for several years and the results extensively reviewed (2,3,9,19,33,34,35,36,37). The biosynthesis and interconversion of polyamines in mammalian cells is shown schematically in fig 1.2. Polyamine metabolism is a cyclic process which allows the interconversion of putrescine, spermidine and spermine according to physiological requirements. This so called "interconversion pathway" is essential for the regulation of cellular polyamine turnover (1,38). In most cases the requirements of cells for polyamines cannot be satisfied by putrescine until it is converted to spermidine (37).

1.3.2 Polyamine biosynthesis

The polyamine synthetic pathway consists of four essentially irreversible steps (Fig 1.2). Three key enzymes regulate polyamine synthesis; ornithine decarboxylase (ODC), S-adenosylmethionine decarboxylase (AdoMetDC) and acetyl CoA:spermidine-spermine N^1acetyl-transferase (C-SAT). The short half-lives of these enzymes, which range from 20 to 40 minutes, allow their activity to be regulated by changes in the amount of active enzyme with dramatic responses to induction within a few hours (2). The polyamine synthetic pathway is also regulated by the availability of ornithine.

Polyamines regulate their own formation and degradation. Spermidine and spermine are believed to be important in the physiological regulation of ODC activity and high levels of these polyamines result in a profound decline in the synthesis of ODC protein. In mammalian cells, sustained high levels of putrescine may induce the synthesis of a protein called antizyme (39) which binds

Fig 1.2 The polyamine metabolic cycle
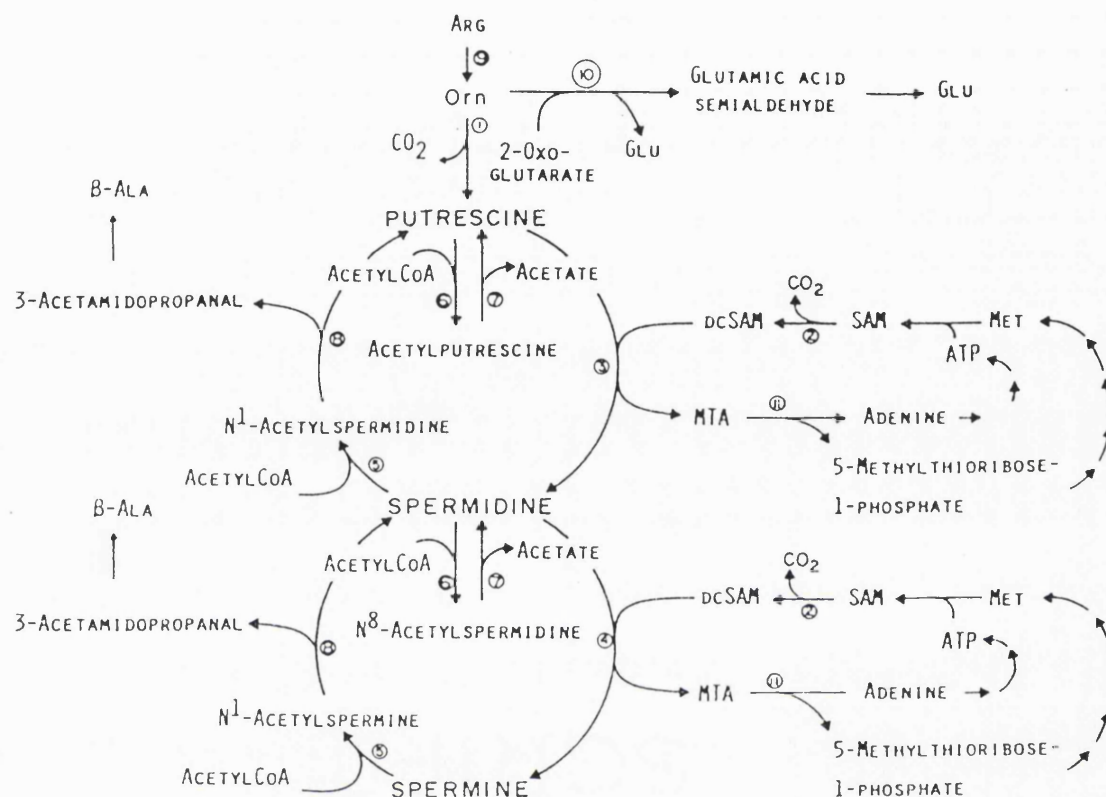
(1) L-Ornithine decarboxylase (ODC) ; (2) S-Adenosyl-L-methionine decarboxylase (AdoMetDC) (3) Spermidine synthase ; (4) Spermine synthase ; (5) AcetylCoA:spermidine/spermine N^1-acetyltransferase (cytosolic) (cSAT) ; (6) AcetylCoA:spermidine N^8-acetyltransferase ; (7) N^8-Acetylspermidine deacetylase (cytosolic) ; (8) Polyamine oxidase (flavin dependent) (PAO) ; (9) Arginase ; (10) L-ornithine:2-oxoglutarate aminotransferase (OAT) ; (11) 5'-Deoxy-5'-methylthioadenosine phosphorylase.

Arg = L-arginine ; Orn = L-ornithine ; Glu = glutamic acid Met = methionine ; β-Ala = β-alanine ; SAM = S-adenosyl-L-methionine ; dcSAM = decarboxylated S-adenosyl-L-methionine (S-3-aminopropyl-5'-methylthioadenosine) ; MTA = 5'-deoxy-5'-methylthioadenosine.

21

to, and inactivates ODC (40), possibly by stimulating its breakdown (41). An inactivator of antizyme or "anti-antizyme" has also been purified (42).

ODC activity is increased, usually by an increase in synthesis, in response to hormones, growth peptides and regenerative stimuli (43). It has been suggested that the transcriptional induction of ODC may be mediated by the formation of cyclic AMP following, for example, hormonal stimulation and the activation of a cyclic AMP-dependent protein kinase (44,45,). Polyamines may play a regulatory role by altering the binding of the hormone to the receptor and therefore inhibiting the formation of cyclic AMP (44). This may be mediated following the interaction of the polyamines with a receptor site. Interaction with this receptor may also induce antizyme formation. In addition, ODC may be partially regulated by stabilization of existing ODC, for example by asparagine and glutamine, which may increase the half life of the enzyme (46).

AdoMetDC levels are also elevated in response to growth promoting stimuli and this may be the result of translational regulation. In addition, physiological levels of putrescine increase the rate of formation of AdoMetDC from a larger proenzyme form (47). Putrescine is also an allosteric regulator of AdoMetDC (48).

1.3.3 Polyamine catabolism

Polyamine catabolism is mediated by a number of routes (49). One is oxidative deamination which is catalyzed by Cu^{2+} containing polyamine oxidases localized in peroxisomes (1). The end products of these reactions are aldehydes, hydrogen peroxide and ammonia which are termed terminal catabolites since they cannot be reconverted into polyamines (37). The aldehydes are usually further metabolised to amino acids, but in the absence of suitable aldehyde dehydrogenases, the

chemically reactive aldehydes may react in various ways.
For example, from the aldehydes derived from spermidine
and spermine, the rather toxic acrolein may be formed by
spontaneous ß-elimination (2)(fig 1.3).

Fig 1.3   The formation of acrolein
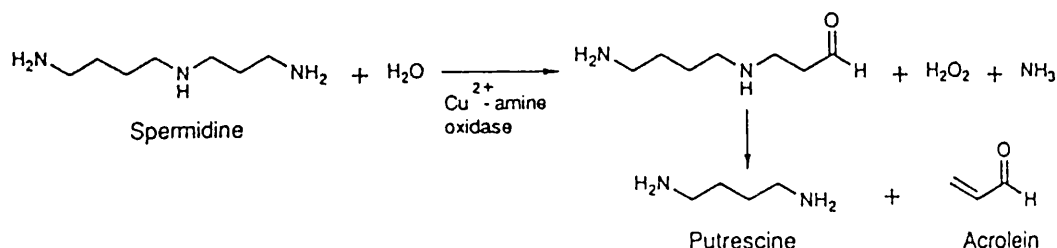
Mono-acetylation of polyamines also takes place
and in vertebrates this is catalyzed by cytosolic and
nuclear N-acetylases. Acetylation removes a positive
charge, decreasing the electrostatic interaction of the
polyamines with negatively charged binding sites.
Polyamines are also more transportable in this less
charged form (50). The only route of N^8-acetylspermidine
metabolism is deacetylation. However, N^1-acetylated
polyamines are oxidized by polyamine oxidases (50).
Their oxidation can result in the production of
putrescine which can be re-utilized in the biosynthetic
pathway, excreted by cells (51) or oxidised by diamine
oxidase. When radiolabelled spermidine is administered
to rats it is acetylated and then converted to putrescine
by this pathway (52).

The acetylase/oxidase pathway may be a regulatory
response that acts to reduce spermidine content when it
becomes too high. However, many toxic agents induce
spermidine/spermine N^1-acetyltransferase resulting in the
formation of N^1-acetyl derivatives which can be oxidized
to toxic hydrogen peroxide. Cells have a differential
ability to protect against the damage caused by this
agent, by means of glutathione (53).   It has even been

23

suggested that the acetylation and oxidation of
polyamines to hydrogen peroxide may play a role in
programmed cell death (54,55).

1.4 The polyamine uptake system

1.4.1 Introduction

Although intracellular polyamines are largely the
product of a tightly regulated biosynthetic pathway, an
increasing number of cell types, both prokaryotic (Tabor
1984) and eukaryotic (56)(table 1.1) have been shown to
possess a polyamine uptake system, which under suitable
conditions can substitute for *de novo* synthesis (56,57).
Polyamine uptake has also been demonstrated *ex vivo* in a
number of intact tissues including rat lung slices
(58,59,60), human lung slices (61), mouse cerebral
hemispheres (62) and rat cerebral cortex slices (63).
The polyamine uptake system is an active process,
specific for polyamines and their structurally related
analogues (56,64).

The mechanisms by which polyamine uptake is
induced are not known but it is clear that polyamine
uptake is normally low in quiescent cells or cells that
have been induced to differentiate (65,66,67). This is
in contrast to cells in rapid growth, for example small
intestine and tumours, where both uptake and synthesis
are enhanced (56,65)(table 1.1). Uptake of
extracellular polyamines is also enhanced by growth
factors and hormones (68,69,70,71) as well as by
inhibition of intracellular synthesis (56,72,73,74).
Polyamine uptake is reduced in cultured cells which have
reached confluency (71) and is accompanied by a reduction
in growth rate. Byers and Pegg suggest that polyamine
uptake may also provide a salvage function for polyamines
(75). The free polyamine pool is probably important in

Table 1.1 Cells possessing a polyamine uptake system

CELL TYPE	REFERENCE
L1210 (murine)	77
EATC (murine)	78
NB 15 neuroblastoma (murine)	79
LoVo colon adenocarcinoma (human)	51
B16 melanoma (murine)	80
BHK cells (transformed)(hamster)	81
AR4-2J (pancreatic carcinoma)(rat)	82
SV3T3 transformed fibroblast (murine)	83
fibroblast (human)	71
C6 glioma (murine)	64
U251 glioma (human)	64
lymphocyte (human)	84
lymphocyte (bovine)	85
erythrocyte (human)	86
LLC-PK$_1$ renal epithelial (porcine)	87
hepatocyte (mouse)	88
hepatocyte (rat)	56
ovary (chinese hamster)	75
enterocyte (rat)	89
pulmonary epithelial type II (rat)	90
pulmonary alveolar macrophage (rabbit)	91
mammary gland (mouse)	69
adrenocortical (bovine)	92
vascular endothelium (human)	76
Girardi heart cells (human)	93
embryonic palate mesenchyme (murine)	94

regulation of polyamine uptake (76).

One of the earliest studies on the postulated polyamine uptake system was undertaken by Field et al, who were investigating the accumulation of the spermidine analogue methylglyoxal-bis(guanylhydrazone)(MGBG) (fig 1.1) into normal and leukaemic human leucocytes, L1210 mouse ascites tumour cells, HeLa cells, rabbit reticulocytes, frog and skate erythrocytes, but not mammalian erythrocytes (95). MGBG uptake was found to be saturable and sensitive to changes in temperature and metabolic poisons. Putrescine, spermidine and spermine competitively inhibited MGBG uptake indicating that they utilize the same transport system. Similarly, in studies into the accumulation of MGBG (96) and the putrescine analogue, paraquat (58,60) into rat lung slices and paraquat into human peripheral lung slices (61), the presence of putrescine was found to have an inhibitory effect.

Since these studies, the presence of a saturable, temperature dependent polyamine uptake system, capable of accumulating polyamines against a concentration gradient, has been demonstrated in many cell types (table 1.1). The saturable nature of the polyamine uptake system suggests that it is carrier mediated. However the source of energy used in the uptake process is not clearly defined since the uptake of the polyamines and MGBG show varying sensitivity to metabolic inhibitors such as rotenone or iodoacetate in combination with KCN. (59,60,62,63,69,97,98,99). Particularly inconsistent results with cyanide are reported for MGBG (95), leading to the conclusion that the most probable energy source for MGBG uptake is glycolysis (95). In contrast, it is reported that the uncoupling of oxidative phosphorylation by 2,4-dinitrophenol in endothelial cells profoundly inhibits polyamine uptake, suggesting that oxidative phosphorylation is an important energy source for

polyamine uptake (76).

The importance of polyamine uptake varies in
different cell lines (64,100). For example, in a
mutant CHO cell line (A7) unable to synthesize
ornithine due to a deficiency in arginase, uptake of
exogenous polyamines (or ornithine) is required for
growth (100). In addition the dependency of cells on
intracellular polyamine biosynthesis is directly
related to their capacity to accumulate polyamines from
the environment. For example, polyamine biosynthesis
inhibitors are more efficient in mutant L1210 cells
which lack the capacity to meet their demand from the
environment (101). Another interesting observation is
the role of polyamine uptake in the differential
toxicity of polyamine analogues to cells. Byers *et al.*
suggested that resistance of a mutant CHO cell line
(CHOMG) to MGBG and paraquat was due to reduced
polyamine uptake in this variant compared to the
sensitive CHO cell line which possessed the uptake
system (75,102). Differences in MGBG accumulation are
also seen in sensitive and resistant murine melanoma
cells (103).

1.4.2 General characteristics of polyamine uptake

The majority of cells appear to have a single
transporter for putrescine, spermidine and spermine, as
judged by polyamine uptake competition studies
(64,69,70,94). The ability of a single carrier to
transport more than one polyamine has been confirmed by
Byers et al (104) who, by transfection of a single
gene, induced the transport of putrescine and spermine
in a polyamine transport deficient CHO cell line.

The affinity for the polyamine transporter
increases from putrescine to spermidine and then
spermine with published Km values in the micromolar
range (56). However, there is also evidence that, at

least in some cells, more than one pathway for
polyamine uptake exists (56)(Table 1.2). For example,
in human endothelial cells, putrescine uptake is
inhibited by spermidine and spermine, but putrescine
itself does not inhibit spermidine and spermine uptake
(76). The existence of multiple carriers with
overlapping substrate specificity has also been
proposed previously (82,85,89,102,105,106) and each
transporter is reported to have different affinities
for putrescine, spermidine and spermine (87,89,92,102).

There are discrepancies concerning the sodium
dependent nature of polyamine transport and this may be
due to varying combinations of these two classes of
transporters (102). In LLC-PK$_1$ renal epithelial (87),
bovine adrenocortical (92), LoVo (51), CHO (102) and
B16 melanoma cells (80) polyamine transport is reported
to possess both Na^+-dependent and Na^+-independent
components.

Putrescine transport in a number of cell types
is reported to be at least partially Na^+-dependent
(76,80,89,92,106). This partial Na^+ dependency has been
interpreted as indicating the presence of two
transporters for putrescine (87,107). Data relating to
the Na^+ dependency of spermidine uptake is more variable
with Na^+ dependence in pneumocytes (106), enterocytes
(108), pulmonary epithelial cells (90) and vascular
endothelial cells (76) but not in adrenocortical (92)
and B16 melanoma (80) cells. In contrast to putrescine
and spermidine, spermine transport is generally Na^+-
independent (76,92,106) (table 1.2). In addition, Na^+-
independent transport of spermine in adrenocortical
cells is not saturable (92).

Van Den Bosch et al. found that, at least in
LLC-PK$_1$ renal cells, Na^+-dependent uptake occurs
preferentially on the baso-lateral side, whereas Na^+-
independent uptake occurs to the same extent on either
the basolateral or apical side (107).

28

TABLE 1.2 Characteristics of polyamine uptake in different cell lines

Cell type	Km (µM)			Transporter	Energy dependence	Effect of		Interaction with amino acid transport systems	Reference
	Put	Spd	Spm			NaCl	Thiols		
Fibroblast (human)	1.1	ND	ND	Single	Yes	ND	ND	No	71
									70
Enterocyte (rat)	12.3	ND	ND	Multiple	Yes	No effect	ND	No	89
Mammary gland (mouse)	ND	48	ND	Single	Yes	ND	ND	No	69
Hepatocyte (rat)	ND	ND	20	ND	Yes	Activation	ND	ND	109
Lymphocytes (human)	ND	ND	ND	Multiple	Yes	Activation	ND	ND	84
Lymphocytes (bovine)	3.7	0.38	0.23	Single	Yes	Activation	ND	ND	85
Adrenocortical (bovine)	10	ND	ND	Multiple	Yes	(a) Activation (b) No activation	ND	No	92
Pulmonary epithelial type II (rat)	ND	0.5	ND	Single	Yes	Activation	ND	No	90
									106
Embryonic palate mesenchymal (mouse)	5.8	ND	ND	Single	Yes	Activation	Activation	No	94
Macrophage pulmonary alveolar (rabbit)	2.2	0.2	ND	Single	Yes	ND	ND	ND	91
Ovary (Chinese hamster)	6.2	6.3	1.0	Multiple	Yes	ND	ND	No	102
	6.9	3.9	1.4	Multiple	ND	ND	ND	ND	75
L1210 Leukemia (mouse)	8.5	2.2	1.6	Single	Yes	ND	ND	ND	77
	ND	2.5	ND	Single	Yes	Activation	No effect	Yes	64
B16 Melanoma	4.1	1.3	ND	Multiple	Yes	ND	ND	ND	56
	5.36	ND	ND	Multiple	Yes	(a) Activation (b) No activation	ND	ND	80
NB15 Neuroblastoma (mouse)	2.8	ND	ND	Single	Yes	Activation	Activation	Yes	67
									79
LLC-PK1 Renal epithelial (1990)	4.7	ND	ND	Multiple	Yes	(a) Activation (b) No activation	Activation	No	87
									107
Morris hepatoma (rat)	155	2.3	1.0	Single	Yes	ND	ND	No	48
Erythrocyte (human)	ND	ND	ND	Single	Yes	Activation	No	No	86
	37	1.6	ND	ND	Yes	ND	ND	ND	110
U251 glioma (human)	ND	1.0	ND	Single	Yes	Activation	Activation	No	64
Pancreatic tumour (rat)	3.1	0.42	ND	Multiple	Yes	No effect	ND	ND	82
Vasular endothelial	3.0	0.7	1.0	Multiple	Yes	Activation	ND	ND	76
EATC	ND	3.0	ND	Single	Yes	Activation	ND	ND	78

ND = not determined

The functioning of the Na^+/K^+ pump might be correlated with spermidine internalization since ouabain, which inhibits the pump, also inhibits spermidine uptake (111). Spermidine and Na^+ are also transported with 1:1 stoichiometry in 3T3 cells (112). In isolated rat enterocytes, a carrier mechanism separate from that of putrescine appears to exist for spermidine and spermine since the uptake of spermidine, but not putrescine, is inhibited by ouabain (108) . In this study, spermidine transport was Na^+-independent since sodium could be replaced by other cations without affecting transport. It is apparent that maintenance of a certain membrane potential, established by Na^+/K^+ ATP-ase, is essential for polyamine uptake (64,108) and it is concluded that this may contribute to a possible source of energy required for the transport process (111).

The transport of cationic amino acids, termed the system A pathway, is also dependent on extracellular Na^+ concentrations (113). In some cells, polyamine transport is influenced by system A amino acids (79,94) while in others uptake is completely independent of these molecules (85,90). It is assumed that in one cell type only one of these transport systems will exist. In most mammalian cells, polyamine uptake is independent of system A transport (56).

The activation of transport by thiols (glutathione, dithiothreitol etc) and inhibition by thiol reagents (p-chloromercuribenzene sulphonate, N-ethylmaleimide) has been studied by several investigators (79,86,87,94). The results show that in all cells the Na^+-activated transporter requires thiol groups in order to maintain its active configuration. A further interesting observation is that the non-specific binding of polyamines to carboxylic groups of sialic acid residues in murine leukemia cells inhibits the concentration of substrates of the polyamine uptake

system at the site of the transporter (114,115). Their removal by desialation results in increased spermidine uptake (115).

1.4.3 Structural specificity of the polyamine uptake system

The mechanism of transport of MGBG into L1210 cells (116) and polyamine analogues into erythrocytes (117) depends on the charge on the species. Charged molecules are taken up by carrier mediated transport and non-charged species by diffusion. Lysyl-L-lysine, a molecule comparable in size and charge with spermidine and spermine, does not inhibit polyamine uptake in vascular endothelial cells (76) suggesting that molecular size and charge alone are insufficient to ensure binding to the transporter, and that some degree of stereospecificity must be involved.

In addition to spermidine, spermine and putrescine, a number of polyamine homologues and analogues can also be transported by the polyamine uptake system (77,97,102,118,119,120).

The structural specificity of compounds utilizing the polyamine uptake system has been investigated using polyamine uptake competition studies. The rationale behind this is that compounds which utilize the same uptake system competitively inhibit each others uptake (56,60,75,95,120,121). The primary amine groups appear to be more critical for uptake than the secondary amine groups (122). N-alkyl substituents on the terminal amino groups of putrescine (123), spermidine (97,121,121) or spermine (124) decrease the ability of the derivatives to compete with polyamines for uptake (56). In contrast, N^4-alkyl derivatives of spermidine are still good substrates for the uptake system, with even a benzyl group substituent being a better substrate than MGBG (97,121). N^4-acyl

derivatives are not good inhibitors (122).

In L1210 cells, triamines are better inhibitors than diamines (77) and maximum inhibition is achieved by those amines having chain lengths resembling spermidine or spermine. These observations are in line with the assumption that the recognition site of the transporter contains (at least) three negatively charged groups separated by a distance corresponding to the distance between the positively charged N atoms of spermidine, presumably in its most stable all-trans configuration.

A study using bi-, tetra- and hexa-pyridinium quarternary salts also shows that the number of positive charges and the distance separating them influences their potency in inhibiting polyamine uptake (125). Increasing the number of charges results in increased potency, suggesting that a greater number of ionic interactions with the recognition site of the transporter occur with increasing numbers of quarternary nitrogens. Consequently it is implied that the recognition site is comprised of several negatively charged domains to which the substrate binds. Results from this study also show that the inter-nitrogen distance of 0.6-0.7 nm or 1.0-1.1nm is optimal for high activity (125).

In rat lung slices, a distance of up to four methylene units (0.66nm) between cationic nitrogen containing groups is required for a compound to be a suitable substrate for the pulmonary diamine and polyamine uptake system. In addition, carbonyl groups and increasing methylation at sites adjacent to cationic amines inhibit their affinity for the uptake system (126,127).

Paraquat (fig 1.1) also utilizes the lung polyamine uptake system. In their research on the mechanism of the specific toxicity of paraquat to the lung, Smith et al. have shown that paraquat is taken up

by both human and rat lung by what appears to be an
active uptake system (61,128) similar to that
responsible for the uptake of putrescine
(58,60,61,129). The structure of paraquat (a
quaternary bipyridyl) (fig 1.1) satisfies many of the
criteria outlined above as necessary for utilization of
the uptake system. Paraquat is also reported to enter
HL60 cells by a temperature dependent polyamine uptake
system (130). In B16 melanoma cells and isolated type
II pneumocytes paraquat is reported to be accumulated
by at least one but not all of these uptake systems
(102,131).

1.4.4 Regulation of polyamine uptake

The mechanism by which polyamine uptake is
increased following, for example, hormonal stimulation
is unclear. It is reported that stimulation of
putrescine transport in murine embryonic palate
mesenchymal (MEPM) cells by epidermal growth factor is
not dependent on transcription of new mRNA or on the
translation of existing mRNA. It appears that
biochemical alterations of the carrier protein which
result in greater efficiency, rather than the synthesis
of increased numbers of receptors, is responsible for
the increased putrescine transport (68).

Protein kinases may play a role in polyamine
transport regulation (115,132). The cellular responses
to extracellular agonists are, in general, mediated by
specific receptors and often result in alterations of
either cyclic AMP (cAMP), calcium, inositol
polyphosphates or diacylglycerol. These intracellular
messengers exert their effects by mechanisms which
often involve covalent phosphorylation of appropriate
rate limiting enzymes, ion channels or other proteins
(133). Three multifunctional protein kinases, cAMP-
dependent protein kinase (cAMP-kinase), calcium-

phospholipid-dependent protein kinase (protein kinase C) and calcium/calmodulin-dependent protein kinase II (CaM-kinase II) are thought to mediate many of these phosphorylation reactions (134).

CaM-kinase II may be involved in the regulation of putrescine uptake since, in PC-3 prostatic cells, inhibition of CaM-kinase II by calmodulin antagonists reduces putrescine uptake (132). In this study inhibition of cAMP kinase and protein kinase C did not inhibit putrescine uptake. In contrast, protein kinase C may be important in the regulation of spermidine transport since uptake is decreased by specific inhibitors of protein kinase C in L1210 cells (115). Further evidence for the role of protein kinase C in spermidine transport is provided by the observation that phorbol ester mediated stimulation of spermidine uptake in L1210 cells is mediated by protein kinase C activation and is accompanied by a stimulation of the Na$^+$/K$^+$ pump (111). It can therefore be argued that transport processes operated for spermidine are different from those for putrescine (115). This may explain why the reported affinity of normal erythrocytes for spermidine (Km 3.6μM) is higher than that for putrescine (Km 125μM) (86,110,114). Furthermore, in a mutant CHO cell line with reduced catalytic activity of cAMP kinase, uptake of spermidine and putrescine was reduced five fold compared to normal cells (135). These findings suggest that all 3 protein kinases may play a role in polyamine transport regulation.

The activities of these enzymes, sometimes designated the `messenger dependent' protein kinases, can, in certain circumstances, be affected by polyamines but the effect is usually inhibitory (16,68). This could provide a negative feedback mechanism whereby when polyamines reach a certain intracellular concentration, further polyamine uptake

is inhibited. Alternatively, polyamines may play a regulatory role by altering hormone-receptor binding (44). In tumour cells, where polyamine uptake occurs in the abscence of hormonal stimulation, the ability of polyamines to regulate their own uptake (and synthesis) may be altered (44).

1.5 Polyamines in transformed cells and tumours

It has been well established that polyamines accumulate in tissues undergoing rapid and neoplastic growth (3,19,33,43,136). An elevation of the spermidine : spermine ratio is also indicative of cells undergoing proliferation and is reported in transformed fibroblasts (3T3 cells) compared to non transformed cells (83), EATC (137), L1210 tumour cells (138) and in regenerating liver (136).

Elevated intracellular polyamine levels can result from increased biosynthesis and/or uptake from an exogenous source. High polyamine levels are also seen in the plasma of tumour bearing rodents (139,140) and humans, possibly resulting from their release from dead and dying cells (2).

Alterations in polyamines have been implicated in tumour promotion and carcinogenesis and increased ODC activity, possibly by stabilization of the enzyme, is an early event related to the transition from normal to neoplastic growth (33). Tumour cells may also lack the receptor involved in ODC regulation (44).

Transfection of rat-1 cells with the ras-oncogene results in a twelve-fold increase in ODC activity (124). This rise in ODC activity is thought to be connected to the rapid growth phase associated with tumour promotion. In contrast, transfection of rat-1 cells with the N-myc oncogene results in a twenty-six fold increase in the rate of polyamine uptake but no change in synthesis. These observations

suggest that the alterations in polyamine synthesis and uptake that occur in transformed cells are regulated by different genes (124).

Further examples of the effects of transformation on polyamines include the observation that infection of mouse kidney cell cultures with polyoma virus (an oncogenic DNA virus) causes a biphasic increase in the activities of ODC and AdoMetDC as well as intracellular levels of the three main polyamines (22). In transformed fibroblasts (SV 3T3 cells) there is an alteration in ODC regulation which results in high intracellular concentrations of ODC and subsequent accumulation of putrescine and spermidine (83). Infection of cultured chick embryo fibroblasts with Rous sarcoma virus also results in sustained increased ODC activity (141) and putrescine levels (142).

In transformed baby hamster kidney cells, elevated polyamine levels appear to be the result of both increased biosynthesis and increased polyamine transport (81). Increased putrescine uptake is also reported in AR4-2J (pancreatic carcinoma) cells compared to isolated rat pancreatic acini which do not possess a putrescine uptake system (66). The lack of a transport system for putrescine in normal rat panreatic cells may be explained by the observation that the acetylase/oxidase pathway is a common source of putrescine in these cells (143). It is also reported that a DFMO-resistant rat hepatoma cell line, in addition to being an over producer of ODC, also lacks the normal mechanism for feedback control of polyamine uptake (144).

Differences in putrescine uptake are also reported in undifferentiated mouse neuroblastoma cells compared to differentiated cells (67) and in rat gliosarcoma compared to normal surrounding brain (145). Putrescine administration to DFMO pretreated mice

innoculated with the EMT 6 tumour results in a tumour selective accumulation of putrescine (146). Pretreatment of L1210 tumour infected mice with α-DFMO has also demonstrated that selective uptake of MGBG occurs into the tumour cells with little effect on other tissues, generally resulting in a synergistic response at doses of MGBG causing less host toxicity (147,148).

In addition to the growth promoting effects of polyamines on tumours, their oxidation products, such as acrolein, have growth inhibitory effects on neoplastic cells (149). Thus polyamines may contribute to the control of primary tumour growth and metastasis.

1.6 Interference with polyamine biosynthesis and/or function as a potential anticancer chemotherapeutic strategy

Polyamines are recognised as being indispensible for cell proliferation. The ability to successfully manipulate the differences in polyamine metabolism and uptake in normal and tumour cells would provide a powerful therapeutic tool (65). Limitation of the access of tumours to polyamines has therefore been seen as a logical step towards a new strategy of cancer chemotherapy (121).

The two common approaches to this are the inhibition of polyamine biosynthetic enzymes and the inhibition of polyamine uptake into tumour cells by polyamine analogues/homologues which themselves may also have regulatory effects on the biosynthetic enzymes.

1.6.1 Inhibition of polyamine biosynthetic enzymes

The rate limiting enzymes ODC and AdoMetDC together with spermidine/spermine synthase originally appeared to be suitable targets for inhibition. However, resistance to inhibitors of polyamine biosynthesis is common and attributable to the fact that cells exposed to antimetabolites activate a series of compensatory mechanisms that prevent the level of at least one of the polyamines from decreasing (150). Although several inhibitors of polyamine biosynthesis are currently available (Pegg 1988), only two have gained therapeutic significance; the ODC inactivator α-(difluoromethyl)ornithine (DFMO) (fig 1.4) and methylglyoxal-bis(guanylhydrazone) (MGBG)(fig 1.1).

1.6.1.1 α-Difluoromethylornithine (DFMO)

DFMO (fig 1.4), a structural analogue of ornithine, is an enzyme activated, irreversible inhibitor of ODC (2) which enters cells by diffusion (151). Inhibition of ODC results in a gradual depletion of spermidine and putrescine and a decrease in the rate of cell proliferation in a variety of cells (12,100,152). Spermine levels remain unaltered (72,150).

Fig 1.4 Structure of α-DFMO

$$H_2N\left(CH_2\right)_3 \underset{\underset{CHF_2}{|}}{\overset{\overset{COOH}{|}}{C}}-NH_2$$
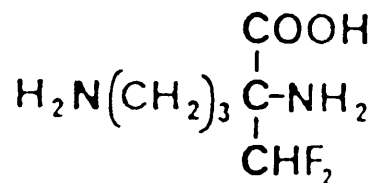
It is well established that intracellular polyamine depletion can result in enhanced uptake of exogenous polyamines to restore levels (56). This is

especially apparent in cells with a high de novo rate of polyamine synthesis such as rapidly growing tumours; for example EATC (72), prostate tissue (73) as well as the intestinal mucosa and bone marrow (74). Enhanced polyamine accumulation also results in the increased uptake and subsequent toxicity of polyamine analogues, as demonstrated by MGBG in EATC (72,147,153,154).

DFMO is usually cytostatic rather than cytotoxic (155), since growth inhibition can generally be reversed by the addition of exogenous polyamines to cells *in vitro* (11). *In vivo*, polyamines released into the circulation from the gastrointestinal tract (2,156,157) or from dead or dying cells (2) can act as a source for polyamine replenishment. As a result, the activity of DFMO as an antitumour agent is somewhat limited. However, DFMO is reported to prevent metastasis of tumours (158) and, as it demonstrates no inherent host toxicity may be useful in this respect (2).

Cells treated with DFMO tend to accumulate in specific phases of the cell cycle. *In vitro*, most normal cells arrest in G_0 or early G_1 following polyamine depletion with DFMO (159). In contrast to normal cells, most transformed cells arrest in S phase (159).

When used in conjunction with other drugs, DFMO may result in increased or decreased toxicity depending on their cell cycle specificity (160,161,162). In rat 9L brain tumour cells polyamine depletion inhibits the G_1 to S transition (163). There is also an alteration in DNA conformation (164) which may be responsible for their decreased radiosensitivity. A reduction in the *in vitro* toxicity of aziridinyl-benzoquinone to polyamine depleted 9L cells is also reported (165). In contrast, studies with nitrosourea compounds suggest that DFMO enhances the toxicity of those agents which alkylate and cross-link DNA but reduces or has no

effect on the toxicity of those which carbamoylate only
(166). Further evidence suggests that the increased
toxicity of BCNU and cis-platinum drugs is not due to
increased cross-linking of DNA but of some other target
(161,167,168)

1.6.1.2 Methylglyoxal-bis(guanylhydrazone) (MGBG)

The ability of MGBG to utilize the polyamine
uptake system has already been described
(72,95,96,120,169). MGBG may act as a non-functional
structural analogue of spermidine and is a competitive
and reversible inhibitor of AdoMetDC (37,170,171). A
comprehensive review of the actions of MGBG is provided
by Williams-Ashman and Seidenfeld (172).

Inhibition of AdoMetDC, which occurs with
micromolar concentrations of MGBG (171), results in a
depletion of intracellular spermidine and spermine.
Putrescine levels, however, remain unchanged (119) or
even slightly elevated (173). This polyamine depletion
by MGBG actually stimulates the polyamine uptake system
(119) and, in EATC, stimulates further uptake of MGBG
resulting in a 1000-fold concentration gradient of MGBG
across the cell membrane (72,154). This is followed by
an inhibition of cell growth (72,147,153). Polyamine
depletion with DFMO also increases MGBG accumulation
(72,147,153). This suicide accumulation is unique for
MGBG and does not occur with spermidine which only
accumulates until it reaches normal levels
(72,119,153).

The cell growth inhibition caused by MGBG is
probably not solely due to polyamine depletion since
the millimolar concentrations of MGBG that can be
achieved in cells (154) can also directly inhibit
mitochondrial oxidative phosphorylation. The
mitochondrial effects of MGBG include the observation
that MGBG causes ultrastructural changes in the

mitochondria of intestinal epithelium *in vivo* (174).
In addition, the lysis of the outer membrane of rat
liver mitochondria results in increased mitochondrial
uptake of MGBG and enhances the inhibitory effects on
mitochondrial respiration (175).

MGBG appears to have two different and distinct
effects on mitochondria and polyamines since
mitochondrial damage precedes significant alterations
in polyamine pools (176,177). Also, polyamine
depletion by other agents such as α-DFMO does not cause
mitochondrial damage. However, disruption of
mitochondrial spermine levels by MGBG may affect
oxidative phosphorylation since small fluctuations in
spermine levels are believed to play a role in
mitochondrial metabolic regulation *in vivo* (178).
Therefore the mitochondrial effects of MGBG could be
polyamine related.

An interesting observation in MGBG resistant 9L
cells is that there are no conspicuous mitochondrial
ultrastructural alterations on exposure to the drug
(179). In addition, in contrast to the data of
Pleshkewych *et al.* (177), significant ultrastructural
changes in the mitochondria of 9L cells treated with α-
DFMO are reported (179).

It has been demonstrated that the sensitivity
of variants of the human VA$_2$ cell line (180), murine
melanoma (103) and L1210 cells *in vitro* (120) and in
L1210 cells *in vivo* (181) depends both on their ability
to accumulate MGBG and to maintain levels after
accumulation, since in the less sensitive cells MGBG
efflux occurs following uptake. This suggests that
the underlying mechanism of MGBG toxicity is dependent
on certain internal levels of MGBG being maintained,
possibly by intracellular binding.

MGBG possesses some *in vivo* activity against
rodent tumours, especially if used in combination with
DFMO (148), although with the exception of sarcoma 180

ascites, all sensitive tumours are leukemias. The drug is inactive against solid sarcomas and carcinomas (182). However, recently developed inhibitors of AdoMetDC, which are structurally related to MGBG, are reported to have activity against solid B16 melanoma (183).

A review by Porter (184) summarizes the clinical and experimental developments with MGBG, but in general its clinical usefulness has been limited by its narrow therapeutic index which is apparent both in rodents and man (182,185). The activity of MGBG against acute myelocytic leukaemia and malignant lymphomas is counteracted by severe toxicity (186), findings which have been confirmed repeatedly. The dramatic side effects of MGBG in man are mainly gastrointestinal toxicity and bone marrow depression, as well as hypoglycaemia, general infection and acute respiratory failure (182). These side effects are seen in a number of species (182,187) and are presumably due to the disposition of MGBG in the affected tissues (73,74,148,181,188), possibly via the polyamine uptake system.

1.6.2 Inhibition of polyamine uptake and/or function and enzyme regulation.

The antitumour activity of a variety of polyamine analogues has been investigated. In addition to inhibiting polyamine uptake, many polyamine analogues also have a high affinity for intracellular binding sites, due to their increased electrostatic charge, and may displace the natural polyamines. (10,118,189). If the polyamine analogues are non functional this results in toxicity (189). For example, spermine analogues with a higher affinity for DNA than spermine, but with less ability to bend or

condense DNA are reported to have growth inhibitory effects (190). Other studies demonstrate that the activity of tetramine homologues depends on their methylene chain length and is possibly due to differing DNA interactions (118).

In addition to these effects, high intracellular concentrations of polyamine analogues, their metabolites and the polyamines they displace may inhibit biosynthetic enzymes (117,191,192) and induce acetyl transferase/oxidase pathway(193,194,195,196). This can lead to the depletion of functional polyamines. This effect is reported in L1210 cells with N^1,N^8-bis(ethyl) spermidine (10,197) where inhibition of cell proliferation, similar to that seen with DFMO, occurs. In addition, toxic polyamine metabolites may also be formed from the acetylated polyamines (193).

Good *in vivo* activity against L1210 cells by bis(ethyl)spermine deivatives (198) and with tetramines derived from 1,8-diaminooctane (199) has been reported. More recently, the activity of N^1,N^{11}-bis(ethyl)-norspermine against human melanoma xenografts has been described (194). A possible explanation for the good activity of these compounds may be that they act in a similar manner to bis(ethyl)spermidine, the growth inhibitory effects of which are not accompanied by an increase in polyamine uptake (10). This is in contrast to DFMO which stimulates a compensatory increase in polyamine uptake.

Studies have shown that some cells, even from the same organ, are more susceptible to polyamine analogues than others. Undifferentiated small cell lung carcinoma (non SCLC), which is resistant to DFMO, is more sensitive to bis(ethyl)-spermine derivatives than SCLC (which is sensitive to DFMO)(191).

1.7 Alkylating agents

The types of alkylating agents used clinically include nitrogen mustards, aziridines, alkylalkane sulphonates and nitrosoureas. As a class, the alkylating agents share a common target (DNA) and are cytotoxic, mutagenic and carcinogenic (200).

1.7.1 Mechanism of alkylation

Traditionally, alkylating reactions have been classified as S_N1 (nucleophilic substitution, first order), or S_N2 (nucleophilic substitution, second order) (fig 1.5). In an S_N1 reaction there is an initial formation of a highly reactive intermediate, followed by the rapid reaction of this intermediate with a nucleophile to produce an alkylated product. In contrast, the S_N2 reaction represents a bimolecular nucleophilic displacement. S_N2 reagents are less reactive than S_N1 reagents. Clinically useful agents include drugs which alkylate through both S_N1 and S_N2 mechanisms (200).

The general mechanism of alkylation utilized by bifunctional alkylating nitrogen mustards is shown schematically in fig 1.6. In the initial step, chlorine is lost and the ß-carbon reacts with the nucleophilic nitrogen atom to form the cyclic, positively charged and very reactive aziridinium moiety. Reaction of the aziridinium ring with a nucleophile (electron rich atom) yields the initial alkylated product. Formation of a second aziridinium ring by the remaining chloroethyl group allows for a second alkylation which provides a cross link between the two alkylated nucleophiles (200).

Fig 1.5 S_N1 and S_N2 reactions

S_N1
$$RX \longrightarrow R^+ + X^- \xrightarrow{+ Y^-} RY + X^-$$

S_N2
$$RX + Y^- \longrightarrow [X....R....Y] \longrightarrow X^- + RY$$
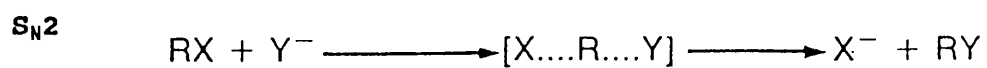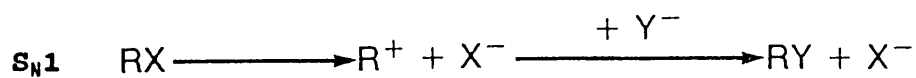
Fig 1.6 Mechanism of alkylation by nitrogen mustard

R—N
CH₂ CH₂ Cl
CH₂ CH₂ Cl   NITROGEN MUSTARD

Cl

R—N⊕—CH₂
CH₂ CH₂ Cl
CH₂   IMMONIUM ION

[R—N
CH₂ CH₂ Cl
CH₂
CH₂⊕]   CARBONIUM ION

NUCLEOPHILIC CENTER   CH₂—CH₂—N⊕—CH₂
R
CH₂   SECOND IMMONIUM ION

[SECOND CARBONIUM ION]

CH₂—CH₂—N—CH₂—CH₂
R
    BIFUNCTIONAL CROSS LINK

45
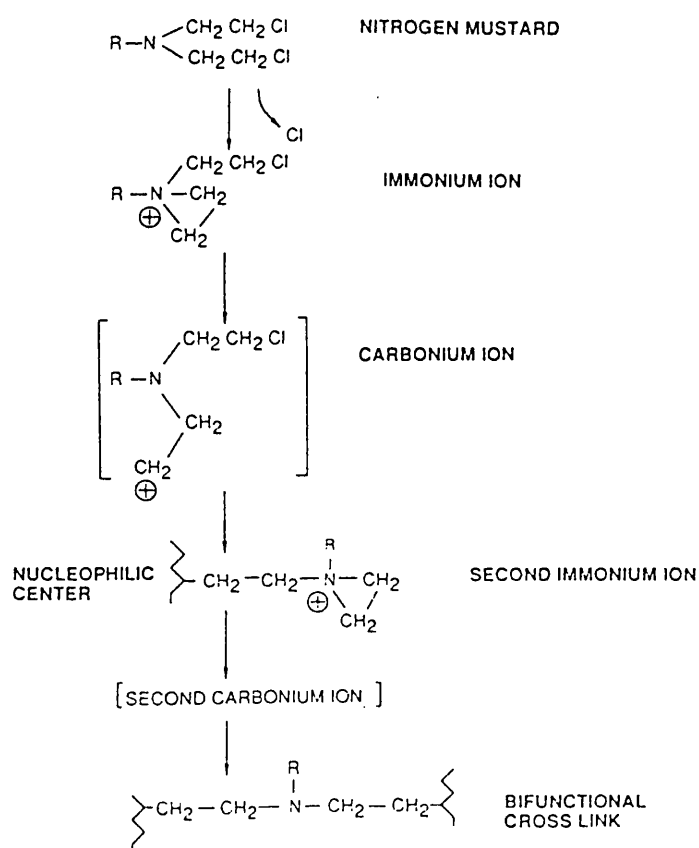
1.7.2 Mechanism of toxicity

It is generally accepted that the cytotoxicity
of alkylating agents is due to the inactivation of
critical macromolecules such as nucleic acids. Since
one of the consistantly observed biochemical effects of
alkylating agents at cytotoxic levels is the inhibition
of DNA synthesis (201,202) a number of studies have
focused on this inhibition. Conflicting reports have
appeared, but evidence favours the hypothesis that
inhibition is due to damage to the nucleic acid
template, rather than inactivation of DNA-polymerase or
other enzymes responsible for DNA synthesis
(201,202,203,204,205,206). ·

It is reported that transcriptionally active
regions of nucleosomal DNA are more susceptible to
alkylation and less easily repaired than linker regions
(207). It is now generally accepted that the precise
mechanism of bifunctional agent activity is the
formation of lethal DNA cross links (fig 1.7) following
alkylation of guanine residues at the N^7 position in GC
rich sequences (208,209,210,211,212) which results in
cell death.

Fig 1.7 Cross-linking of DNA by nitrogen mustard
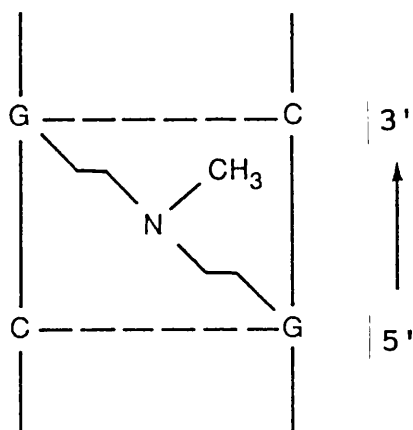
The DNA sequence specificity of many alkylating agents is due to the variation in electrostatic potential at the guanine N^7 position in GC rich sequences. The accessibility of guanine N^7 does not appear to be an important factor (209).

The suggestion that formation of DNA cross links is important in the mechanism of action of bifunctional alkylating agents is also supported by the fact that the bifunctional alkylating agents, with few exceptions, are much more effective antitumour agents than the analogous monofunctional agents, as originally described by Loveless and Ross (200,213). Studies have shown that DNA interstrand cross linking by aniline mustards, as measured by alkaline elution, correlates with *in vitro* cytotoxicity in human (LS 174T) colonic adenocarcinoma and leukemic (K562) cells (214). In addition, cross-linking does not occur in some drug resistant cells (215). The order of activity of the aniline mustards can also be predicted by their ability to produce cross-links in isolated DNA (214).

In addition to DNA crosslink formation, which results in the inactivation of the DNA template and subsequent inhibition of DNA synthesis and imbalanced growth (216), other mechanisms for the toxicity of alkylating agents have been suggested. Therapeutic concentrations of alkylating agents such as Trenimon (aziridinyl-benzoquinone) are reported to alkylate and cause extensive alterations in plasma membranes resulting in decreased activity of carrier mediated processes, including thymidine transport (217,218). Confirmation of plasma membrane effects is provided by the observation that conjugation of Trenimon to a macromolecular carrier, which presumably does not enter the cell, still results in an antitumour effect (218). Trenimon may also uncouple DNA and histone synthesis by activation of non-specific nuclease and offers an explanation as to why low concentrations of alkylating

agents can give growth arrest in the G_2 stage of the cell cycle (218). Other studies suggest that low concentrations of nitrogen mustard inhibit membrane Na^+/K^+ ATPase activity in mouse ADJ/PC6A plasmacytoma cells (219).

An interesting study demonstrates that adsorption to red blood cell membranes by a number of alkylating agents, including chlorambucil, occurs by virtue of the fat solubility of the chloroethyl group and could cause cell lysis at high concentrations (220). Alkylation is not involved but lipophilic charge is important. However, at therapeutic concentrations, lysis is not evident and binding to red blood cells in this manner may protect the active group of the drug from attack by nucleophilic substances in the blood and provide a transport mechanism.

Other suggested mechanisms of alkylating agent activity include inhibition of a low Km form of cyclic 3',5'-nucleotide phosphodiesterase which causes an increase in cyclic AMP levels and growth inhibition (221). The contribution of the high affinity form of the enzyme to the total amount is greater in cells sensitive to the alkylating agents than in resistant cells.

1.7.3 Metabolism

Most alkylating agents also undergo some degree of enzymic metabolism. A major route of metabolism of chlorambucil is oxidation of the butyric acid side chain to produce phenylacetic acid mustard (PAAM) (222,223,224). PAAM, which itself is an active alkylating agent, undergoes hydrolysis to produce the mono and bishydroxyethyl products (222,223,224). In addition, chlorambucil also undergoes enzymic and non-enzymic conjugation to glutathione, which results in its deactivation (225,226).

48

Most of the reactive alkylating agents also undergo spontaneous hydrolysis of the alkylating entity (ie alkylation of H_2O). For example, chlorambucil will undergo hydrolysis to form the monohydroxyethyl and bishydroxyethyl products (200).

1.7.4 Alkylating agent resistance

The emergence of alkylating agent resistant tumour cells is a major problem that limits the clinical effectiveness of these drugs. Although decreased drug uptake is an obvious mechanism of cellular drug resistance (227,228), evidence for other mechanisms of resistance to alkylating agents has been presented. These include increased repair of drug induced damage, increased scavenging of drug species by non-essential cellular nucleophiles, increased enzymic detoxication of drug species and failure to activate prodrugs (225,226,229,230,231,232).

It was reported by Calcutt and Connors in 1963 that tumour cells, resistant to alkylating agents, possessed an increase in thiol content which might function to react with and inactivate the alkylating agent (233). More recently it has been reported that there is an increase in thiol content in melphalan resistant ovarian carcinoma (234) that melphalan toxicity is also enhanced by glutathione depletion (231). Studies indicate that increases in both GSH content and GST activity are evident in a chlorambucil resistant cell line established from the NIH 3T3 mouse fibroblast cell line compared to the sensitive parent cells (230). In addition, an inverse correlation between GSH content and GST activity and chlorambucil induced DNA crosslinks is reported in CHO and chronic lymphocytic leukaemia (CLL) cells (235,236). Aquired alkylating agent resistance is frequently associated with over expression of the GST α isoenzyme which is

the most efficient at conjugation reactions with
chlorambucil (225).

A further potential mechanism to explain
alkylating agent resistance is the enhanced repair of
lesions generated by alkylation. It has been
demonstrated that DNA interstrand cross-links produced
by alkylating agents can be repaired by mammalian cells
(237). In Yoshida sarcoma an increase in the formation
and removal of DNA cross-links is seen in a
chlorambucil resistant variant (229), although no
difference is seen in sensitive and resistant CHO cells
(235). With the exception of guanine O^6-
alkyltransferase in nitrosourea-resistant cells, the
specific enzymes responsible for repair of alkylating
agent DNA lesions have not yet been identified (200).

It is probable that multiple mechanisms of
cellular resistance occur in a given tumour cell
population and are responsible for the drug resistance
seen clinically and with experimental animal tumours
(200). In both situations, varying degrees of cross-
resistance between alkylating agents are seen, but a
tumour which is resistant to one alkylating agent may
remain significantly responsive to another. This
finding forms the rational basis for the use of
combinations of alkylating agents in chemotherapy.

1.7.5 Selective targeting of drugs to tumours

The vast majority of cancer chemotherapeutic
agents in use today have the potential to damage all
proliferating cells. Side effects include
haematopoietic and immuno suppression, pulmonary
fibrosis, gonadal atrophy, gastrointestinal toxicity,
alopecia, nausea and vomiting (200). Several attempts
to selectively target cytotoxics to tumour cells, which
could reduce systemic toxicity, have been made but
these have met with limited success.

50

Covalent binding of chlorambucil to polyunsaturated fatty acids is reported to increase the *in vitro* selectivity of chlorambucil to neoplastic versus quiescent lymphocytes (238). These enhanced activities are thought to be due to the presence of cell surface receptors for α-fetoprotein (AFP), which have a high affinity for polyunsaturated fatty acids and which are present on many tumour cells but not on normal resting cells (238). Conjugates of radioisotopes, antibiotics, alkylating agents, plant toxins and antimetabolites have also been made to monoclonal antibodies raised to tumour specific cell surface antigens (239).

Coupling of drugs to antibodies is difficult and in most cases leads to a severe loss in drug activity although tumour specificity is increased (240). However, for a chlorambucil-ester monoclonal antibody conjugate, which has a high drug to antibody ratio, some *in vitro* and *in vivo* success against ascitic forms of murine thymoma is reported (240). Ricin-antibody conjugates are also reported to have *in vitro* but limited *in vivo* activity (241).

A major problem with antibody targeting is the heterogeneity in the distribution of antigens within the cell population with some cells escaping (242). Some success has been achieved with radiolabelled antibodies (243) which have the advantage in that they can irradiate the cells from outside so internalization is not required. However, i.v. administered antibodies are slow to localize in tumours and much of the radioactivity is delivered somewhat indiscriminately and therefore damages normal tissue.

Many studies involving enzyme activated prodrugs, such as cyclophosphamide, have been performed. In these, activation of latent compounds by enzymes thought to be present in greater concentrations in certain types of cancer than normal tissue was

proposed (244). Unfortunately a favourable distribution of activating enzymes have not been found in human cancers and the prodrugs have not achieved the selectivity that was sought (242). It has been suggested that it may be possible to overcome the limitations of enzyme activated prodrugs by first targeting an antibody linked enzyme to the tumour and localizing it to sites on the cell membrane. Generation of active drug following prodrug administration specifically in the vicinity of the tumour should then be possible (242).

The use of DNA-intercalators as carriers for the delivery of alkylating agents has also been suggested (245). Use of a DNA directed carrier may help reduce drug loss by interaction with other nucleophiles before reaching the DNA and may also help overcome tumour cell resistance which is easily developed by an increase in the levels of low molecular weight thiols (particularly GSH) (246) and DNA repair enzymes (247). In addition, a higher proportion of cross links may occur due to the high affinity of the carrier for DNA. It has even been suggested that medically effective alkylating agents and antimalarial intercalators have polyamine like characteristics, binding at sites on the DNA normally occupied by the polyamines (248,249).

DNA targeting may also help overcome the cell cycle specificity of certain antitumour agents. A consistent pattern has emerged that cells in mitosis or early G_1 are usually more sensitive to alkylating agents (250,251) and this is probably due to less ability at this stage of the cell cycle to repair DNA damage prior to DNA synthesis, as demonstrated by nitrogen mustard (252).

The duration of exposure of cells to cell cycle specific cytoxics is an important determinant of drug induced cytotoxicity, with an increase in cell kill

occuring with increased exposure time (253,254). *In vivo*, the exposure time of tumour cells to drug following administration of a bolus dose is approximately one hour (255), with unreacted drug diffusing back out of the cell as plasma levels decrease. By targeting drugs specifically to DNA the compounds may remain at high concentrations in the vicinity of the DNA even after plasma levels have fallen. DNA targeting may overcome the schedule dependence in a similar manner to continuous exposure or multiple dosing (256). Similarly, the active uptake of melphalan by a leucine carrier system in L1210 cells (257,258), may contribute to its lack of cell cycle specificity compared to chlorambucil which enters cells, including chronic lymphocytic lymphocytes (211) by diffusion. Vistica (258) also suggested that it may be possible to target nitrogen mustard to tumours by conjugating it to a carrier utilizing the leucine uptake system. Cells lacking the transporter should be less sensitive to the drugs in a similar manner to a cell line which lacks the melphalan transporter and is resistant to melphalan (228).

1.8 Polyamine-conjugates as antitumour agents

It has been suggested that N^4-spermidine derivatives might serve as vector molecules for the selective delivery of biologically active groups or small anticancer agents to tumour tissues (97,121). This approach is based on evidence that proliferating cells and cells stimulated to divide take up polyamines to a greater extent than non-proliferating cells (70,71). In addition, differentiated cells take up less polyamines than non-differentiated cells (67).

N^4-alkyl derivatives of spermidine, even those with benzyl group substituents, still have a high

affinity for the polyamine uptake system (97,122). In a similar manner to MGBG, polyamine analogues may be concentrated in relatively high concentrations in neoplastic cells and tissues. However, acyl conjugates, which are poor inhibitors of uptake, are unlikely to be useful with this delivery system (122). In addition to increased uptake, the affinity of the polyamine carrier for DNA should also enhance the potential for target site (ie DNA) interaction (259).

Polyamine conjugates have been tested previously as anticancer agents (260). Two acridine molecules connected by the terminal amines of putrescine, spermidine or spermine could serve as bifunctional alkylating agents. Their primary site of action is RNA synthesis and they have growth inhibitory properties. However, it seems unlikely that they enter cells via the polyamine uptake system since end terminal derivatives are poor substrates of the uptake system. The prevention of cytotoxicity by exogenous polyamines may be related to competition at intracellular sites (121).

Several effective clinical antitumour agents, for example bleomycin, are complex antitumour antibiotics and contain polyamine-amide linked conjugates as part of their complex structure (121). In the case of bleomycin, the polyamine portion is probably important in interactions with DNA rather than uptake.

Deoxyspergualin is a highly active antitumour antibiotic which also contains a polyamine conjugate (121,261,262). It has striking *in vivo* activity against murine tumours. The cytotoxicity of this compound is dependent on its conversion by amine oxidase to an active metabolite (121).

Diam 3, an aziridinyl (Az) bearing cyclophosphazene moiety linked to 1,3-diaminopropane was found to have dose dependent antitumoural effects

54

in mice transplanted with P388, L1210 and P815 cells (263) and in human glioma xenografts (264). It has also been shown that Diam 3 is probably a substrate of the polyamine uptake system of cultured human (U 251) glioblastoma cells (265). Uptake is sodium dependent, system A independent and requires functional sulphydryl groups in a similar manner to polyamine uptake. However, uptake is non-competitively inhibited by polyamines (ie Km and Vmax decreased) (265). The target of the compound remains to be elucidated but toxicity may involve covalent binding.

Aziridinylputrescine (AZP), a cytotoxic putrescine analogue, may also utilize the uptake system to enter human prostatic carcinoma cells (266). The toxicity of AZP is enhanced by DFMO, suggesting that accumulation is enhanced, another characteristic of compounds utilizing the uptake system. However, this increase in toxicity could also be due to the effects of polyamine depletion on DNA stability.

It may be possible to exploit the polyamine uptake system futher still by administering low doses of polyamine-conjugates over a long time period, at plasma concentrations near their Km for tumour uptake (267). This should result in a greater selective uptake into tumour tissue. Possible side effects of polyamine-cytotoxic conjugates include toxicity to other tissues possessing the polyamine uptake system including small intestine (51) and lung (129,267) where the compounds may also accumulate.

1.9 Aims

The overall aim of this study was to attempt to selectively increase the delivery of antitumour agents to tumours possessing the polyamine uptake system. This method of drug targeting has so far met with limited success, partially because the structural requirements for compounds to utilize the polyamine uptake system have not been fully considered. In addition, no attempts to utilize uptake pharmacokinetics (eg Km values) have been made. The aims of this study were therefore to:-

a) Test novel polyamine conjugates for uptake and toxicity to tumour cells and to select those which demonstrated promising activity for further investigation;

b) attempt to utilize pharmacokinetics to further increase the selective uptake and *in vivo* antitumour activity;

c) investigate the role of the polyamine uptake system in the delivery and toxicity of novel polyamine-conjugates to tumours *in vitro* and *in vivo*.

CHAPTER 2 - MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Tumour cell lines

The tumour cells used in this study are described in table 2.1. The human ovarian carcinoma cell lines (PXN-94, SKOV-3, 41M, HX-62) and the murine ADJ/PC6 plasmacytoma were kindly provided by Dr L. Kelland, Institute of Cancer Research, 15 Cotswold Rd, Belmont, Sutton. Ehrlich ascites tumour cells (EATC) were originally obtained by the School of Pharmacy, Univ. of London from Dr M. Jones (Institute of Cancer Research, Sutton) and L1210 cells were purchased from ICN Flow (High Wycombe, Bucks). The cells were maintained *in vitro* and *in vivo* as described (table 2.1).

2.1.2 Cell culture media and supplies

Dulbecco's Modification of Eagles Medium (DMEM), RPMI 1640 medium, foetal calf serum (FCS) and donor horse serum (DHS) were purchased from ICN Flow (High Wycombe, Bucks). PBS "A" tablets were supplied by Unipath Ltd (Basingstoke, Hants). Trypsin/EDTA (x10), penicillin/ streptomycin (x100), gentamycin (x200) and L-glutamine (x100) were obtained from Gibco (Paisley, Scotland). Amphotericin B (x100) was supplied by Imperial (Andover, Hampshire). The growth factors insulin and hydrocortisone were purchased from Sigma Chemical Co. (Poole, Dorset).

All plastic culture vessels and sterile plastic pipettes were obtained from Beckton Dickinson (Cowley, Oxford). Cell culture tubes (75x12mm) (Cell-Cult) were supplied by R.L. Slaughter (Upminster, Essex) and sterile Universal containers (30ml) were purchased from Bibby Sterilin Ltd (Stone, Staffs.). Sterile filters (0.2μm)

Table 2.1   Description of cell lines used in these studies

Cell-line	original source	general morphology in culture	culture medium	culture media supplements	in vivo growth	reference
EATC	mammary gland carcinoma (mouse)	round cells (monolayer)	DMEM 10% FCS	glutamine (1mM) penicillin (50U/ml) streptomycin (50μg/ml)	ascites in peritoneal cavity passage 2x10^6 cells every 7 days	268
L1210	lymphocytic leukemia (mouse)	spheroid (suspension)	RPMI 1640 10% FCS	glutamine (1mM) penicillin (50U/ml) streptomycin (50μg/ml)	ascites in peritoneal cavity passage 1x10^6 cells every 7 days	269
ADJ/PC6	plasma cell tumour (mouse)	spheroid (suspension)	DMEM 15% DHS	glutamine (1mM) hydrocortisone (8μg/ml) insulin (0.3μg/ml) amphotericin B (2.5μg/ml) gentamycin (50μg/ml)	subcutaneous solid tumour passage every 21 days	270
PXN/94 SKOV-3 41 M	ovarian adenocarcinoma (human)	round cells (monolayer) polygonal (monolayer) round cells (monolayer)	DMEM 10% FCS	glutamine (1mM) hydrocortisone (8μg/ml) insulin (0.3μg/ml) amphotericin B (2.5μg/ml) gentamycin (50μg/ml)		271
HX/62	ovarian papillary cystadenocarcinoma (human)	polygonal (monolayer)				

for filter sterilising solutions were supplied by
Sartorius Instruments Ltd (Epsom, Surrey). Sterile
plastic syringes and needles were obtained from Sabre
International Products (Reading, Berks). Cryovials for
storing frozen cells were purchased from Gibco (Paisley,
Scotland).

2.1.3 Animals

Balb/c, DBA/2 and C57/DBA2 F_1 hybrid mice (6-8
weeks) were supplied by Bantin and Kingman (Hull, UK) or
from breeding colonies at the MRC Toxicology Unit,
Carshalton. All animals were housed on a 12 hr
light/dark cycle prior to and during studies.

2.1.4 Chemicals and solvents

2.1.4.1 Test compounds

The nitroimidazole-polyamine conjugates and
chlorambucil-spermidine conjugate were synthesized by Dr
A. Mather (University of Leicester) as described
(272,273). Misonidazole and metronidazole were supplied
by Prof. P. Cullis (University of Leicester).
Chlorambucil and spermidine trihydrochloride were
purchased from Sigma Chemical Co. (Poole, Dorset, UK).
Methylglyoxal-bis(guanylhydrazone) was obtained from
Aldrich Chemical Co (Gillingham, Dorset, UK).

2.1.4.2 Radiolabelled compounds

[6]^{3}H-Thymidine (29Ci/mmol) and ^{14}C-spermidine
trihydrochloride (110mCi/mmol) were purchased from
Amersham International plc (Amersham, Bucks, UK).

2.1.4.3 HPLC reagents

Ammonium acetate, glacial acetic acid, dimethylsulphoxide and perchloric acid (all HPLC reagent grade), methanol (gradient analysis grade) and sodium acetate were purchased from Fisons Scientific Equipment (Loughborough, UK.). Acetonitrile (far UV grade) and sodium chloride (hypersolv) were obtained from BDH Ltd (Poole, Dorset, UK). Trichloroacetic acid (microselect) was purchased from Fluka Chemicals Ltd (Gillingham, Dorset, UK).

2.1.4.4 Other reagents

Aquasol and Monofluor for liquid scintillation counting were purchased from New England Nuclear Ltd (Edinburgh, Scotland) and National Diagnostics (Manville, New Jersey) respectively. Isoton II was obtained from Coulter Electronics Ltd (Luton, Beds., UK). Leishman and trypan blue dyes were supplied by BDH Ltd (Poole, Dorset, UK). p-Iodo-nitrotetrazolium violet, propylene glycol and thymidine were purchased from Sigma Chemical Co. (Poole, Dorset, UK). Absolute alcohol was obtained from Hayman Ltd (Witham, Essex, UK.) and hydrochloric acid from HSA Laboratory supplies (Loughborough, Leic., UK). p-Iodoaniline was supplied by Eastman Organic Chemicals (New York, USA). α-DFMO was kindly provided by the Merrel Dow Research Institute (Strasbourg, France).

2.1.5 Equipment

All sterile procedures were carried out in a Microflow Pathfinder sterile flow cabinet (Inter Med). Cells were maintained in a Leec (model GA2) incubator (Leec Ltd., Nottingham, UK) at 37°C in a 5% CO_2 humidified atmosphere. A Watson light microscope and Zeiss inverted light microscope (Germany) were used to view the cells.

Tumour cells were counted using a Coulter Counter, model
Industrial D (Coulter Electronics, Beds., UK.).

All centrifugations were carried out in a Denley
BR 401 refrigerated centrifuge (Billinghurst, Sussex, UK)
or MSE bench centrifuge. For scintillation counting a
Rackbeta 1216 (LKB Instruments, Surrey, UK.) or Wallac
model 1410 (Pharmacia Diagnostics, Milton Keynes, U.K.)
scintillation counter were used. Tissues were
disintegrated with a Ystral homogeniser (Funkentstort, W.
Germany).

For HPLC analysis a Varian (Walnut Creek, CA,
USA) model 5000 liquid chromatograph was used with a
Varian UV-50 variable wavelength detector. Samples were
injected using a Rheodyne 7125 injector fitted with a
500μl loop.

2.2 METHODS

2.2.1 *In vitro* cell culture techniques

2.2.1.1 Monolayer cultures

EATC and the human ovarian carcinoma cell lines
PXN-94, SKOV-3, HX-62 and 41-M were cultured as
monolayers, using the media described in table 2.1.
Cells were sub-cultured prior to reaching confluence.
This was carried out by aspirating the culture medium
from the flasks using a sterile glass pipette connected
to a vacuum pump and washing the cell monolayers with
prewarmed sterile phosphate buffered saline (PBS)
(2x5ml). 0.05% Trypsin/0.02% EDTA (w/v) (5ml) was added
to the flasks which were returned to the incubator for 5-
10 minutes to allow the cells to detatch from the surface
of the flasks. Prewarmed DMEM/10% FCS (10ml) was added
to each flask and the cell suspensions transfered to 30ml
universals. The cells were pelleted by centrifugation

(1000 rpm, 5min) and the cells resuspended in fresh medium. The cells were counted using the Coulter counter and viability assessed with trypan blue (2.2.1.3). The cell suspension was then diluted in fresh medium to obtain the required seeding density. Cells were seeded into new flasks (approximately 1×10^6 cells per 75cm^2 flask) or used in experiments.

2.2.1.2 Suspension cultures

The murine ADJ/PC6 plasmacytoma and L1210 cell lines grew in suspension. Cells were passaged every 3-4 days by transferring aliqots of cell suspension (3ml) into new flasks containing prewarmed medium (17ml) to give approximately 1×10^5 cells/ml.

2.2.1.3 Cell counting and viability

Cells were counted electronically using a Coulter counter. Aliquots (200μl) of a single cell suspension were added to Isoton II (9.8ml) and the number of particles in 0.5ml of the cell/Isoton mixture counted. To obtain the number of cells per ml of the original cell suspension the particle counts were multiplied by 100.

Cell viability was determined using the trypan blue dye exclusion method. Aliquots (100μl) of cell suspension were gently mixed with 0.4% trypan blue (w/v) in PBS (400μl) in eppendorf tubes. A drop of suspension was placed under a cover slip on a haemocytometer and the cells inspected with a light microscope. The number of cells staining blue (having absorbed the dye and therefore dead) was compared to the total number of cells and from this the percentage viability calculated. The combination of cell density obtained by Coulter counting and percentage viability using trypan blue gave the total number of viable cells/ml.

2.2.1.4 Freezing down tumour cells for storage

Stocks of tumour cells were maintained under liquid nitrogen. These were obtained by trypsinizing monolayer cultures or pelleting suspension cultures and resuspending the final cell pellet obtained in pre-cooled culture medium (4°C) supplemented with 10% dimethylsulphoxide (DMSO) at a density of approximately 10×10^6 cells/ml. Aliquots (1ml) of cell suspension were placed in sterile cryovials and frozen slowly above liquid nitrogen using a freezing tray. The cells were stored under liquid nitrogen until required.

2.2.1.5 Recovery of frozen tumour cells

Frozen cell suspensions were thawed rapidly at 37°C and the cells transferred to universal containers (30ml) containing culture medium (10ml). The cells were centrifuged (1000 rpm, 5 min) to remove the DMSO and resuspended in fresh medium. This was repeated and the final cell pellet resuspended in fresh medium. The cells were transferred to culture flasks and cultured for two weeks (with subculturing as required) prior to their experimental use.

2.2.2 Measurement of ^{14}C-spermidine uptake *in vitro* in the presence and absence of inhibitors

The kinetics of spermidine uptake in the different cell lines was investigated to confirm the presence of a polyamine uptake system. The abilities of the various polyamine analogues to inhibit ^{14}C-spermidine uptake was also studied. Such studies are useful indicators as to whether compounds have an affinity for (and it is assumed enter cells by) the same uptake system(s) (121). Competitive uptake inhibition is characteristic of compounds which utilize the same

carrier, and Ki values give an indication of their relative affinities. The ability of the different polyamine-conjugates to inhibit spermidine uptake was therefore investigated to determine| whether they were possible substrates of the polyamine uptake system. MGBG, a known substrate of the polyamine uptake system, was used as a positive control. Fig 2.1 shows schematically the methods used for measuring ^{14}C-spermidine uptake and its inhibition by polyamine-conjugates/analogues in the monolayer and suspension cultures.

2.2.2.1 Spermidine uptake (monolayers)

Tumour cells (5×10^4 or 1×10^5) were seeded into wells of 24 well multiwell plates in culture medium (0.5ml) and incubated overnight to form monolayers. Prewarmed solutions (37°C) of unlabelled spermidine and any inhibiting compounds being investigated were added to the wells in a volume of 450μl. ^{14}C-Spermidine (50μl) was added to each well and the cells incubated for 30 minutes at 37°C. In some experiments, uptake at 4°C was investigated by precooling solutions and cells and performing the incubations on ice.

Immediately following the incubations the radioactive supernatant solutions were decanted off and the cell monolayers washed three times with ice cold 0.9% NaCl (w/v) (1ml) containing 1mM unlabelled spermidine to displace non-specifically bound label. The cell monolayers were digested overnight in 1M NaOH (300μl) and neutralized with 1M HCl (300μl). Samples were transferred to scintillation vials and Aquasol (3.5ml) or Monofluor (4ml) added. The radioactivity in each sample was measured using a scintillation counter.

Total spermidine uptake into each cell sample was calculated from the ratio of hot:cold spermidine in each incubation mixture. Reciprocal plots of the rate of

Fig 2.1 Measurement of ^{14}C-spermidine uptake and its inhibition by polyamine-conjugates/analogues *in vitro*

Monolayer

Suspension

```
5x10⁴ or 1x10⁵
cells/0.5ml/well
        │
        ▼
Incubate overnight
to form monolayer
        │
        ▼
¹⁴C—spermidine
± inhibitors added
```

```
¹⁴C—spermidine
± inhibitors
0.5ml/tube
        │
        ▼
1x10⁵ cells added
(0.5ml)
```

```
Incubated 30 min
(37°C or 4°C)
        │
        ▼
Radioactive solutions
decanted from cellsᵃ
        │
        ▼
Cells washedᵃ (x3) with 0.9% NaCl
containing cold spermidine (1mM)
        │
        ▼
Cell monolayer or cell pellet
dissolved in 1M NaOH
and neutralized with 1M HCl
        │
        ▼
Samples transfered to
scintillation vials
        │
        ▼
Aquasol (3.5ml) or
Monofluor (4ml) added
        │
        ▼
Radioactivity per sample
measured by
scintillation counting
```

ᵃ Procedure involved pelleting cells by centrifugation to allow removal of supernatant solutions.

spermidine uptake verses spermidine concentration (ie Lineweaver-Burk plots) were used to determine the Km and Vmax of spermidine uptake. The nature of any inhibition and Ki values were determined by analysis of axis intercepts in the presence and absence of test compound. The determination of uptake (and inhibition) kinetics is described in more detail in chapter 3.

2.2.2.2 Spermidine uptake (suspensions)

Solutions of unlabelled spermidine and any inhibitors (450μl) were added to culture tubes. ^{14}C-Spermidine (50μl) was added to each tube followed by cell suspension (2x10^5/ml) (0.5ml). By adding the cells after ^{14}C-spermidine, mixing of the tubes was unnecessary. Incubations were carried out at 37°C or 4°C for 30 min. At the end of the incubation period, the samples were cooled on ice to prevent further spermidine uptake, followed by centrifugation (1000 rpm, 5min, 4°C) to form a cell pellet. The cell pellet was washed three times with ice cold 0.9% NaCl (w/v) (1ml) containing 1mM unlabelled spermidine. Between washes cells were pelleted by centrifugation (1000rpm, 5min, 4°C).

The final cell pellet was digested overnight in 1M NaOH (300μl) and neutralized with 1M HCl (300μl). The radioactivity in each sample was measured by scintillation counting as described before and the Km, Vmax and Ki values were calculated.

2.2.3 *In vitro* cytotoxicity testing

Cultured cells have been useful tools with which to perform initial screens for antitumour activity (274). The most widely accepted assay for measuring antitumour activity is the clonogenic assay which compares the ability of control and treated cells to form colonies (255,275,276). This assay, however, is time consuming and a more rapid method for assessing the *in vitro* toxicity of the polyamine-conjugates/analogues was required. In these studies, therefore, the inhibition of radiolabelled nucleotide (thymidine) into DNA was used to measure *in vitro* toxicity. This assay was chosen for use in these studies in a routine screening context since it is reported to correlate well with the clonogenic assay (271,277,278,279). For compounds which demonstrated promising *in vitro* activity in the thymidine incorporation assay, confirmation was provided using a clonogenic assay.

For the *in vitro* toxicity studies, cells were exposed to a range of concentrations of test compounds for 1, 24 or 72 hr in the appropriate culture medium. Nitroimidazole-polyamine conjugates, chlorambucil-spermidine and the positive control compound, MGBG, were dissolved in DMEM. Chlorambucil and the parent nitroimidazole compounds were dissolved in dimethylsulphoxide (DMSO) and then added to the culture medium at the appropriate concentrations. For these studies, a final DMSO concentration of 0.1% (v/v) was maintained in control and test cultures. All test solutions were filter sterilized prior to their addition to the cells. Cell viabilities were assessed at the end of the incubations by ^{3}H-thymidine incorporation (fig 2.2) or by the clonogenic assay (fig 2.3).

Fig 2.2 Flow diagram of the ^{3}H-thymidine incorporation assay used for *in vitro* toxicity screening

Monolayer   Suspension

| 2.5x10^3 cells/well ± DFMO (0.5mM, 72hr) | or | control cells 1x10^5/well adhere overnight | control cells 1x10^5/tube | or | DFMO pretreated cells (0.5mM, 72hr) 2x10^5/tube |

Equal volume x2 concentrated drug solution (± spermidine) added

1hr incubation with drug + fresh medium (71hr)

24hr incubation with drug + fresh medium (48hr)

72hr incubation with drug

^{3}H-thymidine (0.5µCi) added for final 2hr of incubation

Cells washed[a] (x3) with 0.9% NaCl containing 1mM cold thymidine

10% TCA added to cell pellet/monolayer (4°C, 10 min)

Acid precipitate dissolved in 1m NaOH and neutralized with 1M HCl

Samples transfered to scintillation vials

Aquasol (3.5 ml) or Monofluor (4ml) added

Radioactivity/sample measured by scintillation counting

[a] Procedure involved pelleting cells by centrifugation to allow removal of supernatant solutions

Fig 2.3 Flow diagram of the clonogenic assay

monolayer   suspension

| Day 0
1×10^6 cells ($25cm^2$ flask)
adhere overnight | Day 1
2×10^5 cells/ml
($25cm^2$ flask) |

Day 1
drug added (1hr or 24 hr)

| cells washed, trypsinized,
resuspended in fresh
medium and counted | cells washed[a],
resuspended in fresh
medium and counted |

| Cells seeded into
petridishes
at different densities
(vol 4ml) | Cells aliquoted into
culture tubes at
different densities
(vol 1ml) |

0.32% agarose (3ml)
added to each tube,
mixed and cooled

| Incubated 10 days | Incubated 2-3 weeks |

| Colonies fixed and stained
(with Leishmans)
and counted | Colonies stained (with
p-iodonitrotetrazoliumviolet)
and counted |

Number of colonies obtained
expressed as % control (untreated) cells

[a] Procedure involved pelleting cells by centrifugation to allow removal of supernatant solutions

2.2.3.1 ^{3}H-Thymidine incorporation into cells

Aliquots of tumour cells (2.5×10^3 - 2×10^5 cells) (0.5ml) were added to wells of 24 well multiwell plates (monolayers) or culture tubes (suspensions). Monolayer cultures were allowed to adhere to the surface of the wells overnight. In some experiments a 72hr preincubation with 0.5mM difluoromethylornithine (DFMO) was used to deplete intracellular polyamines. The cells were then exposed to a range of concentrations of test compound for 1, 24 or 72 hr in a final volume of 1ml. The ability of exogenous spermidine to inhibit the toxicity of test compounds was also studied by incubation of cells with spermidine and the test compound at the same time.

Following exposure to the test compounds for the appropriate time periods, the medium was aspirated from monolayer cultures and replaced with fresh medium (1ml). For the suspension cultures, the culture tubes were centrifuged (1,000 rpm, 10 min) and the medium carefully aspirated from the cell pellet. Fresh medium (1ml) was added and the cells were resuspended by gentle agitation. Cells were incubated for a further 71hr (following 1hr exposure) or 48hr (following 24hr exposure) to allow a number of cell divisions to take place and any DNA damage inflicted by the compounds to be manifested as cell death.

Cell viability was assessed by pulsing each well or tube with 0.5 μCi ^{3}H-thymidine (50μl) for the final 2 hr of the incubation period (4hr for HX/62 cells). At the end of the pulse period the medium was decanted from the multiwell plates and the cell monolayers washed three times with 0.9% NaCl (w/v)(1ml) containing 1mM unlabelled thymidine to displace non-specifically bound label. Ice cold 10% trichloroacetic acid (TCA) (w/v) (1ml) was added to each well for 10 min, decanted off and the precipitate formed solubilised in 1M NaOH (300μl). The NaOH digest

was neutralized with 1M HCl (300μl) and the samples transferred to scintillation vials. Aquasol (3.5ml) or Monofluor (4ml) were added to the samples and the radioactivity of each sample measured by liquid scintillation counting.

For the suspension cultures, following the 2 hr pulsing with ^{3}H-thymidine, the cells were pelleted by centrifugation (1000 rpm, 5 min) and the radioactive supernatant decanted off. The cell pellet was washed three times with ice cold 0.9% NaCl (w/v) (1ml) containing 1mM unlabelled thymidine with centrifugation following each wash. Ice cold 10% TCA (1ml) was added to the final cell pellet for 10 minutes followed by centrifugation (3000 rpm, 10 min) to spin down the precipitate formed. The precipitate was solubilized, neutralized and the radioactivity measured as described before.

The percentage inhibition of ^{3}H-thymidine incorporation for each drug concentration was calculated by dividing the amount of radiolabel incorporated into each test sample by the amount incorporated into control (untreated) cells. From this the concentration of drug giving a 50% inhibition of ^{3}H-thymidine incorporation (IC_{50}) was obtained.

2.2.3.2 Clonogenic assay (monolayers)

This method is shown schematically in fig 2.3. Tumour cells (1×10^6) were seeded into 25cm^2 flasks and allowed to form monolayers overnight. Following a 1hr or 24hr exposure to test compound, cell viability was assessed by comparing the ability of control and treated cells to form colonies. Treated monolayer cultures were washed with PBS, trypsinized (2.2.1.1) and resuspended in fresh medium. The cell density of each sample was determined using the Coulter counter and cells were seeded into 5cm petridishes (final volume 4ml) at

different dilutions to yield 40-100 colonies per assay point. Ten days after plating, the colonies were fixed and stained with a methanolic solution of Leishman's stain (1mg/ml). The mean colony counts obtained were expressed as a percentage of control (untreated) cultures.

2.2.3.3 Clonogenic assay (suspension cultures)

Fig 2.3 shows schematically the clonogenic assay method used for suspension cultures. ADJ/PC6 cells (2 X 10^5 cells/ml) were exposed to test compounds for 1hr or 24hr in 25cm^2 culture flasks. Following incubation with test compounds the cells were pelleted by centrifugation (1000rpm, 5min) and the cell pellet washed and resuspended in fresh medium. Cell densities were determined and different numbers of cells aliquoted into culture tubes. The volume in each tube was adjusted to 1ml.

A solution of sterile 0.32% agarose (w/v) in DMEM/DHS was prepared by adding 4% agarose (w/v) (4ml) (melted by heating at 80°C) to DMEM/DHS (46ml). This solution (3ml) was added to each culture tube followed by capping and gentle mixing. The tubes were placed on ice to allow the agarose to form a gel which would maintain the cells in suspension. The cells were incubated for 2-3 weeks until colonies of 1mm diameter were visible. The colonies were stained red by adding a sterile aqueous solution of p-iodonitro-tetrazolium violet (1mg/ml) (400μl) to each tube. Colonies were counted 24 hr after the addition of the dye and the number of colonies obtained for each drug concentration expressed as a percentage of control (untreated) cultures. Low gelling temperature agarose was used for these experiments.

2.2.4 *In vivo* maintenance of tumour cells
2.2.4.1 EATC and L1210 cells

EATC were maintained by weekly passaging in male Balb/c mice (6-7 wks). Mice were innoculated i.p. with $2x10^6$ EATC in a volume of 200μl 0.9% NaCl (w/v). The cells grew as ascites in the peritoneal cavity of the mice and after one week ascitic fluid was withdrawn using a syringe. Cells were counted and diluted in 0.9% NaCl (w/v) to give the appropriate density for the innoculation of further mice.

For the L1210 tumour cells, $1x10^6$ cells were passaged weekly into female DBA/2 mice in a similar manner to EATC.

2.2.4.2 ADJ/PC6 plasmacytoma.

The ADJ/PC6 plasmacytoma was maintained *in vivo* as a solid tumour implanted subcutaneously in the right flank of female Balb/c mice (6-8 wks). The tumour was passaged every 21 days. For this, a mouse bearing the tumour was killed by cervical dislocation and the tumour excised. The tumour was finely chopped into 1mm^3 pieces in sterile saline and one piece implanted, using a sterile Bashford Trochar, into each mouse.

2.2.5 Animal dosing

For the pharmacokinetic, tissue distribution and antitumour studies, mice were dosed either i.p., via a sub-cutaneously implanted osmotic minipump or by continous infusion of drug via a tail vein cannulation.

A variety of vehicles were used for the initial antitumour studies in EATC and L1210 cells (chapter 3). Following these studies a more appropriate vehicle was found for chlorambucil and chlorambucil-spermidine conjugate. For these compounds, 10-fold concentrated

solutions in 0.5M HCl in ethanol were prepared and diluted immediately before dosing with 0.2M K_2HPO_4 in 45% propylene glycol (w/v). A maximum dosing volume of 100μl/mouse was used.

In the experiments where chlorambucil-spermidine conjugate was continuously infused into the dorsal tail vein of mice via a surgically implanted cannula, the vehicle used consisted of 5% ethanol in propylene glycol (v/v). A maximum dosing volume of 130 μl was used. For the osmotic minipump experiments, 5% ethanol in propylene glycol (v/v) was used as the vehicle for chlorambucil-spermidine and 0.9% NaCl (w/v) for MGBG.

In some studies, mice were administered 2% DFMO (w/v) in drinking water for 72hr prior to administration, of test compounds in order to deplete polyamine levels.

2.2.6 Surgical procedures

For some studies, chlorambucil-spermidine was administered to mice by continuous infusion via cannulae surgically implanted in their dorsal tail veins. In addition, in some experiments, MGBG and chlorambucil-spermidine were administered using subcutaneously implanted Azlet osmotic minipumps (Charles River, Margate, Kent).

2.2.6.1 Implantation of tail vein cannulae

Mice were anaesthetised by isofluorane inhalation and remained anaesthetised throughout the procedure. A 1cm vertical incision was made along the skin either side of the dorsal tail vein with a scalpel. A horizontal cut was made allowing the skin flap to be moved aside exposing the vein to be cannulated. Using a disecting microscope, connective tissue was carefully removed and the vein and adjacent artery separated. A piece of

cotton was used to tie off the vein and a small incision
made above the knot. A cannula, prefilled with
heparinized saline and attached to a syringe, was held
by a pair of forceps and gently inserted into the vein.
The cannula was then tied into the vein and a small
amount of saline injected via the cannula to test for
leaks. The cannula was then taped securely into
position. A tail guard was fitted with the cannula
running inside the attatched 20cm spring to prevent
interference from the mouse. Each mouse was placed in an
individual cage with a special runner above it to which
the spring was attached (fig 2.4). This allowed the
mouse some freedom of movement after recovery from
anaesthesia, whilst protecting the cannula.

Syringes containing the dosing solutions were
secured in a Harvard constant rate delivery pump and
cannulae attached to the syringes. Mice were dosed by
continuous infusion at a rate of $10\mu l/hr$, with the
delivery of saline from the dead space of the cannula
occuring for the first 5 hr of the delivery period .

2.2.6.2 Implantation of osmotic minipumps

Osmotic minipumps were filled with the dosing
solution and preconditioned in 0.9% NaCl (w/v) at 37°C for
4 hrs prior to implantation. Mice were anaesthetised
with halothane and a 1cm incision made in the skin in the
mid-scapular region. Scissors were used to separate the
skin from the underlying connective tissue to make a
pocket for the pump. A minipump was inserted slightly
posterior to the scapulae, delivery portal first and the
wound closed with two wound clips. Delivery of compound
began immediately after pump insertion. Pumps were
weighed before insertion and at the end of the delivery
period. If the pumps were working correctly a weight
change was evident.

Fig 2.4 Apparatus "set up" for the continuous infusion of drug to mice via tail vein cannulations
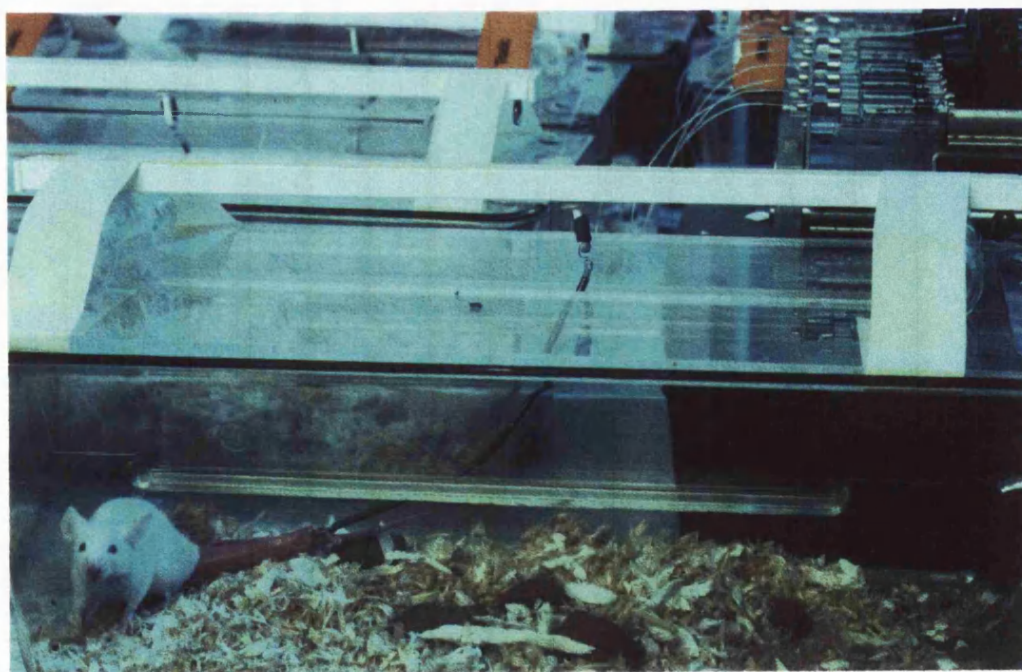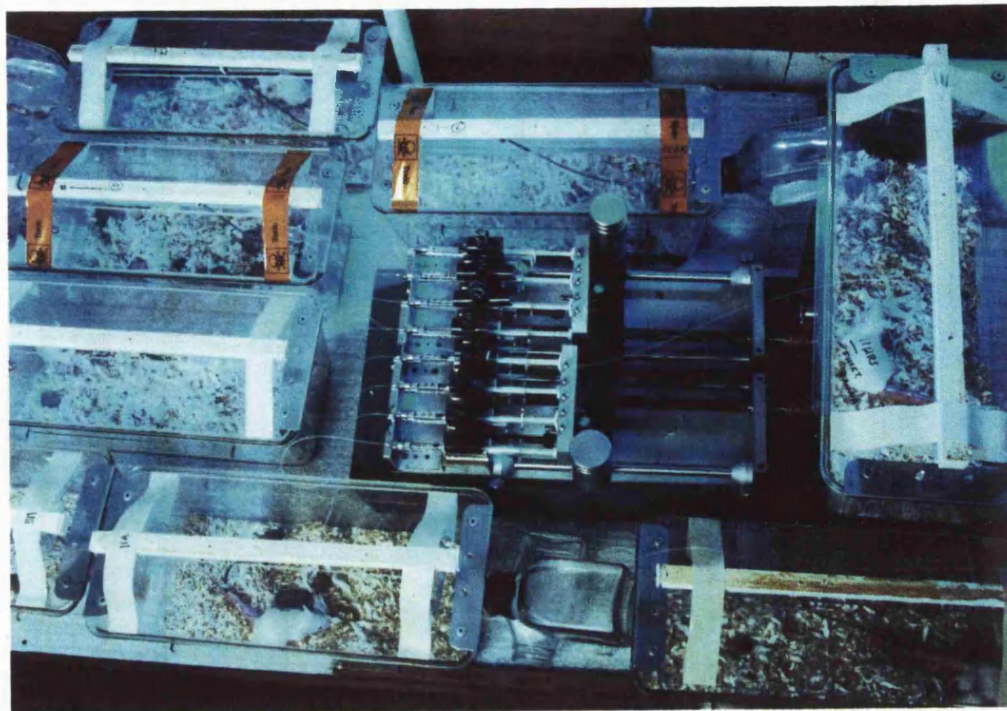
A tail vein cannula was surgically implanted, under anaesthesia, into the dorsal tail vein of each mouse. A tail-guard was fitted and the mice housed in special cages fitted with runners and springs to protect the cannulae. This allowed some freedom of movement whilst protecting the cannulae.

2.2.7 *In vivo* antitumour studies

Cells *in vitro* do not provide unambiguous models for tumours *in vivo* (274) since solid tumours contain cells in a variety of proliferative states and environments. In addition, the kinetics of drug transport to and within tumours and drug metabolism is not as well defined *in vivo* as in cells in culture. The *in vivo* antitumour activity of a selection of compounds which displayed promising *in vitro* activity was therefore investigated. These studies, the methods of which are shown in fig 2.5, were carried out in both ascites (EATC and L1210) and solid (ADJ/PC6) tumours.

2.2.7.1 EATC

Balb/c mice (6-7 weeks) were injected i.p. with EATC ($1x10^6$) ($100\mu l$) on day 0 of the study. On day 1 the mice were randomized into treatment groups of 5 animals and injected i.p with test compound or vehicle. The number of days taken for the mice to become moribund or die as a result of ascites growth was recorded. This was approximately 14 days for control animals and longer for successfully treated animals.

2.2.7.2 L1210

Female C57/DBA2 F_1 hybrid mice (6-7 weeks) were innoculated i.p. with L1210 cells ($1x10^5$) ($100\mu l$) on day 0. On day 1 mice were randomized into treatment groups of 5 animals and dosed i.p. with test compound or vehicle. The time taken for animals to become moribund or die was recorded. This was approximately 20 days for control groups and longer for successfully treated groups.

Fig 2.5 Assessment of the *in vivo* antitumour activity of test compounds

A

Day 0

| Balb/c mice (6–7 weeks) innoculated I.p. with EATC (1x10⁶) | C57/DBA2 mice (6–7 weeks) inoculated i.p. with L1210 cells (1x10⁵) |

Day 1

Mice randomized into treatment and control groups (5/group). Test compounds administered by single i.p. injection

Time taken for mice to die or become morribund recorded Life span of treated animals compared to controls

B

Day 0

1mm³ piece tumour implanted subcutaneously in right flank female Balb/c mice (8 wks)

Day 20

Mice with similar size tumours (1cm³) selected for study : test compound or vehicle administered by single i.p. injection or continuous infusion

Day 29

Animals sacrificed, tumours excised and weighed. Tumour weights compared to untreated (control) animals

Schematic diagram describing the assessment of *in vivo* antitumour activity of compounds in EATC and L1210 cells (a) and ADJ/PC6 plasmacytoma (b).

2.2.7.3 ADJ/PC6 plasmacytoma

In vivo antitumour studies were carried out on the ADJ/PC6 plasmacytoma using a method previously described (280). Small pieces (1mm^3) of ADJ/PC6 tumour (from a freshly excised tumour) were implanted subcutaneously in the right flank of female Balb/c mice (8 weeks old). The tumours were allowed to grow for 20 days, whereupon animals with tumours of a similar size (approximately 1 cm^3) were selected for participation in the antitumour study.

Mice were divided into treatment groups (3 or 4/group) and dosed with either a single i.p. injection or by continuous infusion of freshly prepared dosing solution (2.2.5). Control groups of 6 animals were treated with either vehicle alone or received no treatment. The tumour was allowed to grow until day 29, when all animals were sacrificed by cervical dislocation and the tumours excised and weighed. The mean weight of tumours from each treatment group was expressed as a percentage of the mean weight of the vehicle control group. The doses inhibiting tumour growth by 90% (ED$_{90}$) were calculated and compared. The dose of compound resulting in the death of 50% of animals (LD$_{50}$) was also determined and from this information the therapeutic index (ED$_{90}$/LD$_{50}$) was calculated.

These studies were carried out within the United Kingdom Coordinating Committee on Cancer Research (UKCCCR) guidelines for the welfare of animals in experimental neoplasia.

2.2.8 *In vivo* tissue distribution and pharmacokinetic studies

The pharmacokinetics and tissue distribution of MGBG, chlorambucil-spermidine conjugate and chlorambucil were investigated. Control or tumour (EATC or ADJ/PC6)

bearing Balb/c mice were dosed by i.p. injection,
subcutaneously implanted osmotic minipumps or continuous
infusion and killed at various time points by cervical
dislocation. Tissues were homogenized on ice in 0.9%
NaCl (w/v) and extracted and analysed as follows. Tissue
concentrations were expressed as pmoles/wet weight tissue.

2.2.8.1 MGBG

For the MGBG tissue distribution studies, MGBG
was extracted by adding cold 70% perchloric acid (100μl)
to homogenate (1ml) or EATC suspended in 0.9% NaCl(1ml).
Samples were vortexed and centrifuged (3,000rpm, 10 min,
4°C). The supernatant solutions were transferred to
eppendorf tubes and 10M KOH (100μl) added to each. The
samples were mixed, centrifuged (3,000 rpm, 10min, 4°C)
and the supernatant solutions analysed by HPLC. An
isocratic, reverse phase HPLC system with UV detection at
283nm was used (adapted from 281) to measure MGBG levels.
Sample separation was achieved using a 25cm, 10μm, ODS
column and a mobile phase consisting of 5% methanol in
0.03M sodium acetate (pH adjusted to 4.3 with glacial
acetic acid) (flow rate 1ml/min). Samples were
quantitated by comparing peak areas to those obtained for
standards treated in a similar manner to the samples. A
typical trace is shown in fig 2.6. Tissue concentrations
were expressed as pmoles/wet weight tissue.

Fig 2.6 A typical chromatogram of MGBG
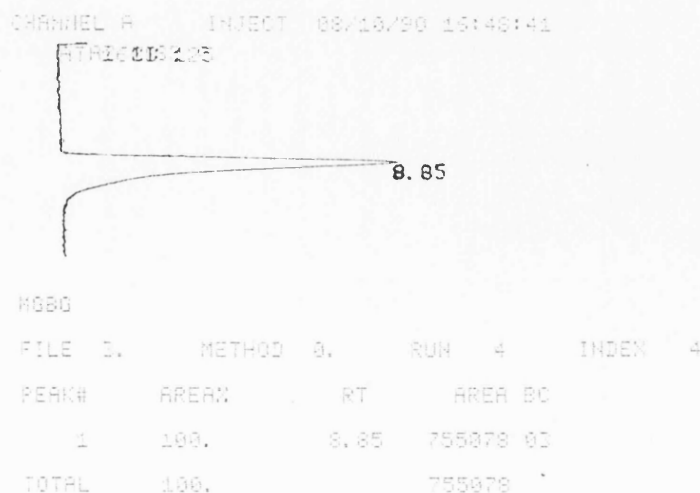
2.2.8.2 Chlorambucil-spermidine and chlorambucil

Concentrations of chlorambucil and chlorambucil-spermidine conjugate in plasma, tissue and ADJ/PC6 plasmacytoma were determined using a specially developed method described in chapter 5. Briefly, plasma, tissue and tumour homogenates were extracted with a mixture of TCA/DMSO containing p-iodoaniline as an internal standard. Samples were analysed using a reverse phase gradient elution method with UV detection at 260nm.

CHAPTER 3 - *In vitro* and *in vivo* activity of a variety of polyamine-conjugates and analogues (including nitroimidazole-polyamine conjugates) to Ehrlich ascites tumour cells.

3.1 Introduction

The aims of these initial studies were to develop and optimize *in vitro* assays for measuring the activity of novel polyamine-cytotoxic conjugates and analogues as antitumour agents. The majority of compounds investigated had been synthesized for use as potential radiosensitizers and were not expected to have any direct cytotoxicity. Nevertheless, the compounds were useful for development of the various assays. In addition, the results obtained gave further insight into the structural characteristics required by compounds in order to utilize the polyamine uptake system. One compound, a chlorambucil-spermidine conjugate, was synthesized specifically as a targeted cytotoxic and its activity was compared to chlorambucil. Three other chlorambucil-polyamine conjugates were also synthesized but were of insufficient purity to warrant detailed investigation.

The polyamine-conjugates and analogues were designed to be potential substrates of the polyamine uptake system. Their ability to utilize the polyamine uptake system may render them selectively toxic to tumour cells. Since a simple assay for measuring the uptake of the individual compounds was not initially available, their potential uptake was assessed indirectly by measuring their ability to inhibit ^{14}C-spermidine uptake into tumour cells. The rationale for this was based on observations that substrates of the polyamine uptake system competitively inhibit each others uptake (56,60,75,95,120,121). It was decided for the initial studies to use EATC since this cell line has a well documented polyamine uptake system (78,119,150,153). It

also has the added advantage that it can be grown both *in vitro* and *in vivo* allowing comparative studies to be performed. MGBG was used as a positive control compound for the spermidine uptake inhibition studies since it inhibits competitively the uptake of spermidine into EATC and is thought to utilize the same uptake system (72,154). In this study, the degree of any inhibition of spermidine uptake was compared for different compounds (by comparison of Ki values) to determine which structural characteristics, if any, rendered compounds good substrates for the uptake system.

The *in vitro* toxicity of the polyamine-conjugates and analogues was also investigated using incorporation of ^{3}H-thymidine into DNA to assess cell viability. For these studies MGBG was again used as a positive control compound. MGBG demonstrates an interesting phenomenon in that its toxicity is greatly enhanced in cells pretreated with DFMO to deplete polyamines (147,153). This is due, at least in part, to enhanced uptake of MGBG into polyamine depleted cells where intracellular concentrations up to 5,000 fold greater than extracellular concentrations can be achieved (72,147). The *in vitro* toxicity of the novel polyamine analogues to both control and polyamine depleted EATC was therefore investigated and compared to the non-conjugated parent compounds.

Many of the compounds studied were nitroimidazole-polyamine conjugates, the structures of which are shown in fig 3.1. Nitroimidazoles, for example metronidazole, are toxic to anaerobic organisms and other anoxic or hypoxic cells. In susceptible cells, the nitro group of metronidazole is reduced by electron-transport proteins and the compound therefore acts as an electron sink, depriving cells of reducing equivalents (282). In addition, the reduced form of the drug may form a DNA base-drug complex which leads to impairment of DNA template function and cytotoxicity (282,283).

Fig 3.1 Structures of the nitroimidazole-polyamine conjugates used in these studies.

84
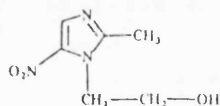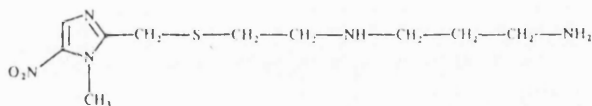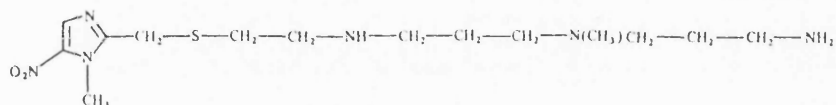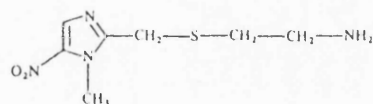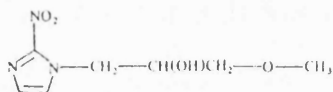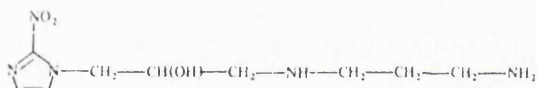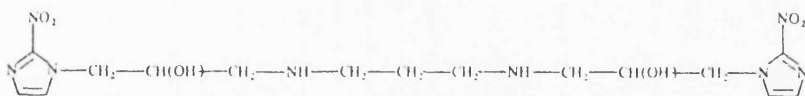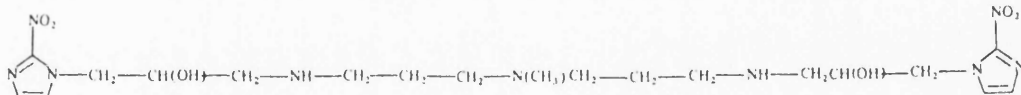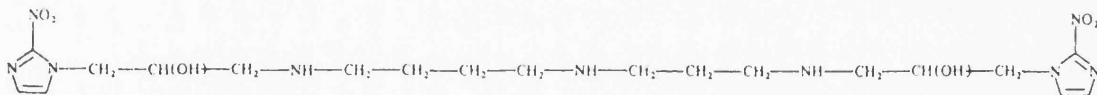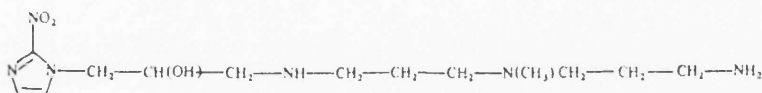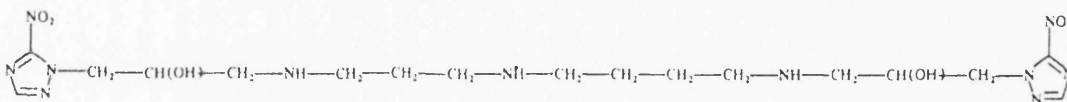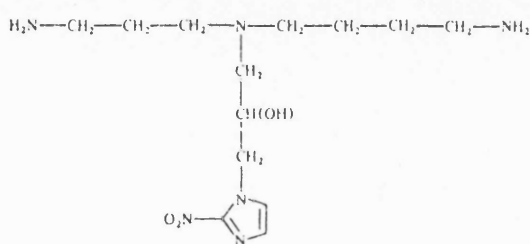
Legend to Fig 3.1 Structures of nitroimidazole-polyamine conjugates used in these studies.

The three figure code used to label these structures is related to the functionality present in the molecules. The first digit represents the class of heterocycle where 1 is equivalent to the 5-nitroimidazoles, 2 to the 2-nitroimidazoles, 4 to the twin 2-nitroimidazoles and 5 to the twin 5-nitrotriazole. The second digit represents the number of polyamine nitrogens and the third digit the number of sulphur atoms present in the molecule. The additional Me represents the substitution of one methyl group on the central nitrogen.

Nitroimidazoles are used clinically as antiprotozoal and antibacterial agents (282). Many nitroimidazoles are also radiosensitizers to hypoxic tumour cells (284). Conjugation to a polyamine may result both in increased uptake into tumour cells via the polyamine uptake system and enhanced targeting of the DNA itself due to the polycationic nature of the polyamine (259). This may result in increased radiosensitivity. Unfortunately, in these studies, the in vitro toxicity of compounds was measured using the ^{3}H-thymidine incorporation assay which did not give any indication of radiosensitization effects. It was therefore not possible to investigate this phenomenon.

Other polyamine analogues were also studied for their ability to inhibit ^{14}C-spermidine uptake. These compounds were not expected to possess any in vitro toxicity to EATC but it was of interest to compare their affinities for the uptake system. This information could possibly help in the future design of the optimal carrier molecule.

Based on the results of these studies, a conjugate of chlorambucil with spermidine (CLB-SPD)(fig 3.2) was synthesized (267). This conjugate was the first in these studies which had the desired characteristics of a targeted cytotoxic in that it consisted of a clinically used cytotoxic agent conjugated to a high affinity carrier molecule. The affinity for the polyamine uptake system (measured by inhibition of ^{14}C-spermidine uptake) and the in vitro toxicity of the CLB-SPD conjugate were compared to chlorambucil. In addition, preliminary in vivo antitumour studies were carried out.

Based on the results obtained with CLB-SPD, further studies were performed with this compound, the results of which are described in the ensuing chapters.

Fig 3.2 Structures of chlorambucil (a), chlorambucil-spermidine conjugate (b), P(I) (N^1,N^8-*bis*(2-hydroxy-2-phenyl-ethyl)-spermidine) (c) and P(II) (N^1,N^8-bis(2-hydroxy-3-phenylether-propyl)-spermidine) (d).

A   B

$H_2N-(CH_2)_4-N-(CH_2)_3-NH_2$
                  |
               $(CH_2)_3$
                  |
                 NH
                  |
                 CO
                  |
               $(CH_2)_3$

COOH
$(CH_2)_3$

C

$-CH(OH)-CH_2-NH-(CH_2)_3-NH-(CH_2)_4-NH-CH_2-CH(OH)-$

D

$-O-CH_2-CH(OH)-CH_2-NH-(CH_2)_3-NH-(CH_2)_4-NH-CH_2-CH(OH)-CH_2-O-$
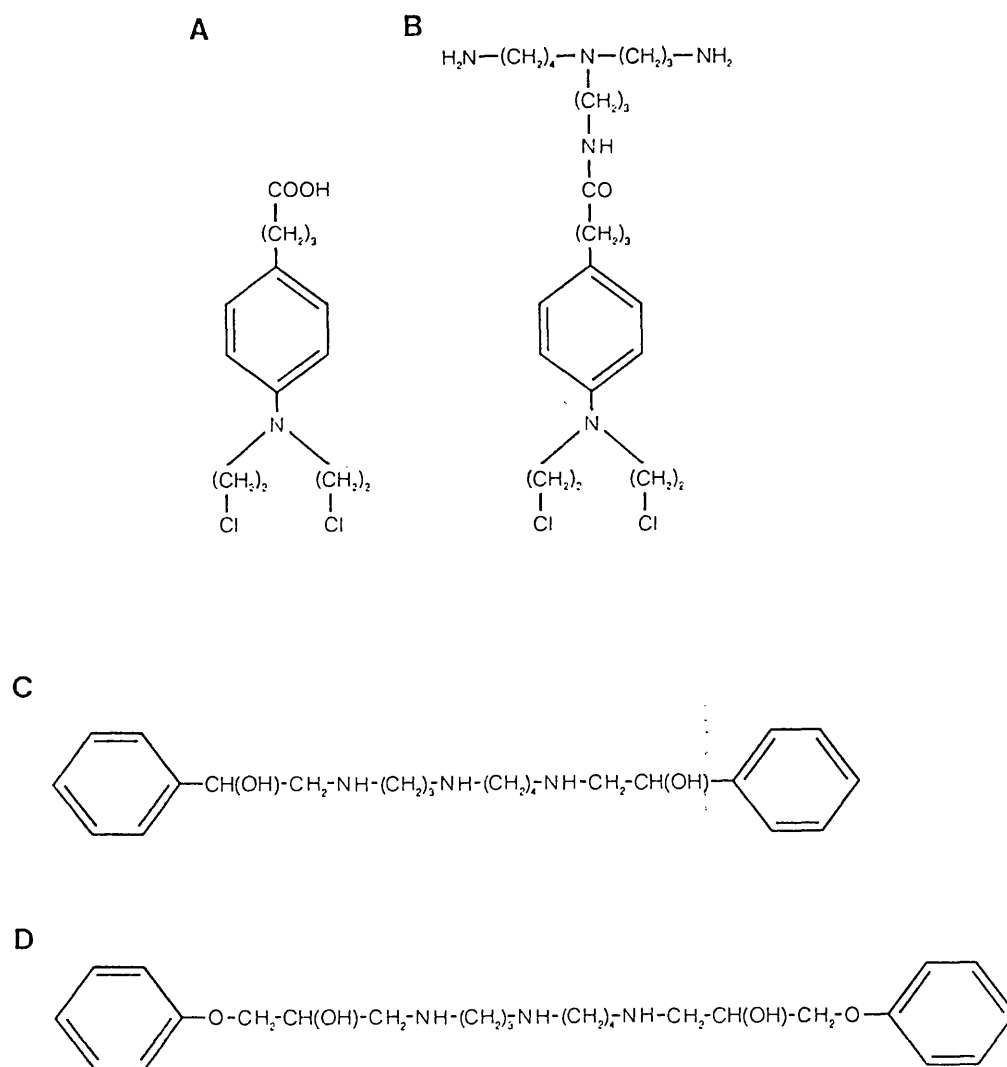
The chlorambucil-spermidine conjugate (b) was synthesized as the hydrochloride salt.

3.2 Results

3.2.1 Characterisation of spermidine uptake in Ehrlich ascites tumour cells; the effect of temperature and polyamine depletion

These initial studies were carried out to confirm that the kinetics of spermidine uptake in EATC were similar to those previously reported in the literature, prior to their use in uptake experiments with the novel polyamine conjugates/analogues. The EATC were found to have a saturable, temperature dependent uptake system for spermidine (fig 3.3). The kinetic parameters for spermidine uptake at 37°C were $Km = 2.2 \pm 0.46$ μM (mean $\pm$ s.e.m)(n=5) and $Vmax = 6.2 \pm 1.1$ pmoles/10^5cells/min (mean $\pm$ s.e.m)(n=5). These were calculated from Lineweaver-Burk (reciprocal rate) plots in the following manner:-

$$Km = \frac{-1}{X\text{-axis intercept}}$$

$$Vmax = \frac{1}{Y\text{-axis intercept}}$$

Pretreatment of EATC with DFMO (0.5mM) for 72 hr to deplete polyamines resulted in an increase in the rate of ^{14}C-spermidine uptake compared to cells preincubated in medium alone (fig 3.4). A 6-fold increase in Vmax was observed. Cells pretreated with DFMO were visibly fewer in number than those incubated in medium alone, although both were subconfluent. Therefore, in this experiment, the rate of spermidine uptake was expressed as pmoles spermidine/mg protein/min to allow for differences in cell number for control and polyamine depleted EATC. The method of Lowry *et al.* was used to measure protein (285).

Fig 3.3   Kinetics of spermidine uptake in EATC;   the effect of temperature.
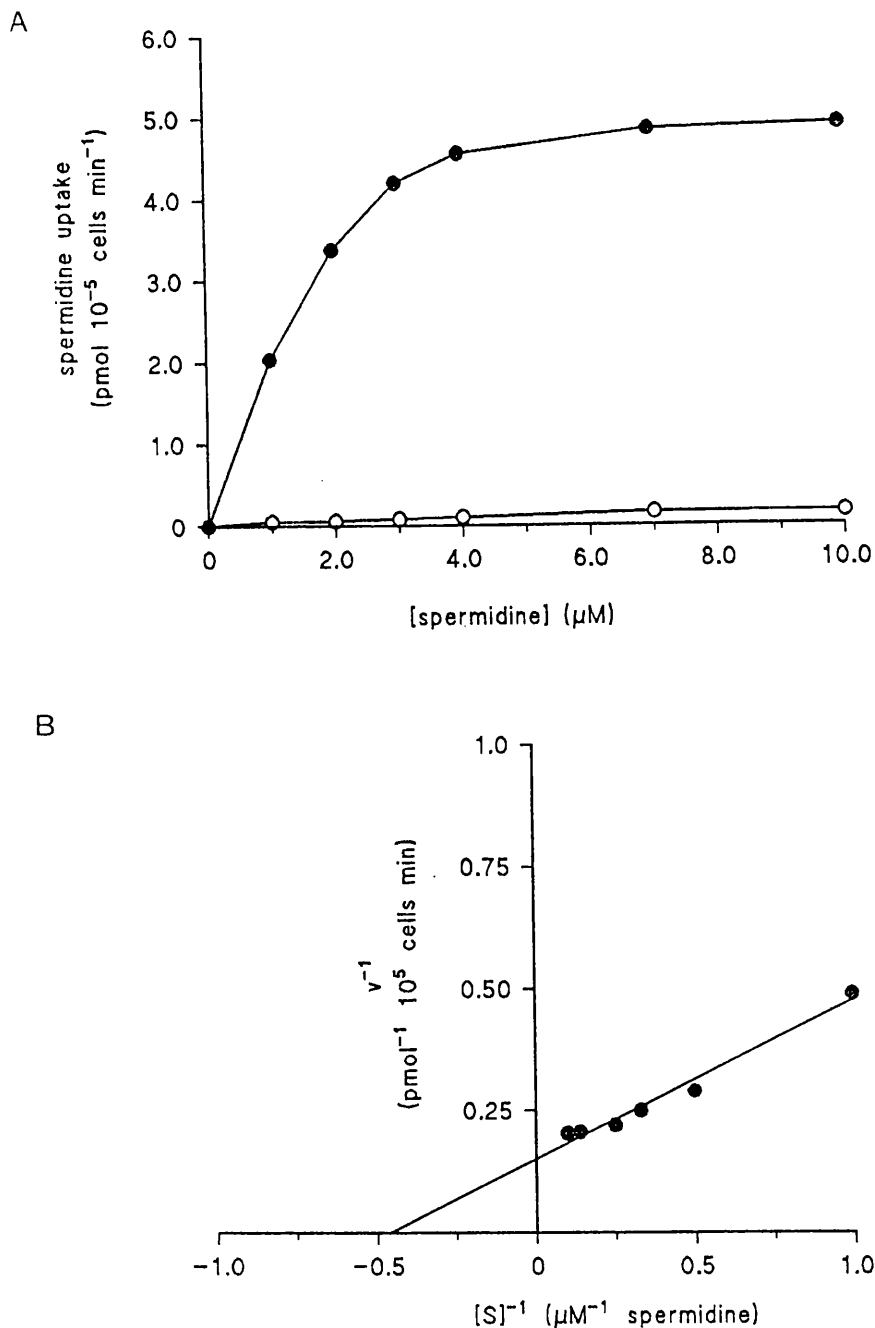
EATC (1×10^5) were incubated for 30 min with ^{14}C-spermidine ($1.0 - 10.0$ μM) at 37°C (●) and 4°C (○). The rate of spermidine uptake is presented as both linear (a) and Lineweaver-Burk (reciprocal rate) (b) plots. Data represent the mean of 5 experiments at 37°C and 2 experiments at 4°C, each performed in duplicate.

Fig 3.4   The effect of DFMO on spermidine uptake in EATC.

A
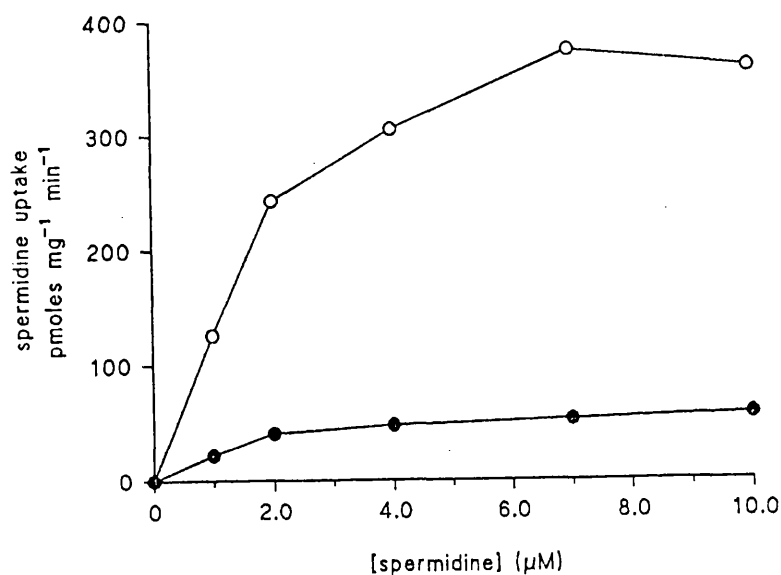
B
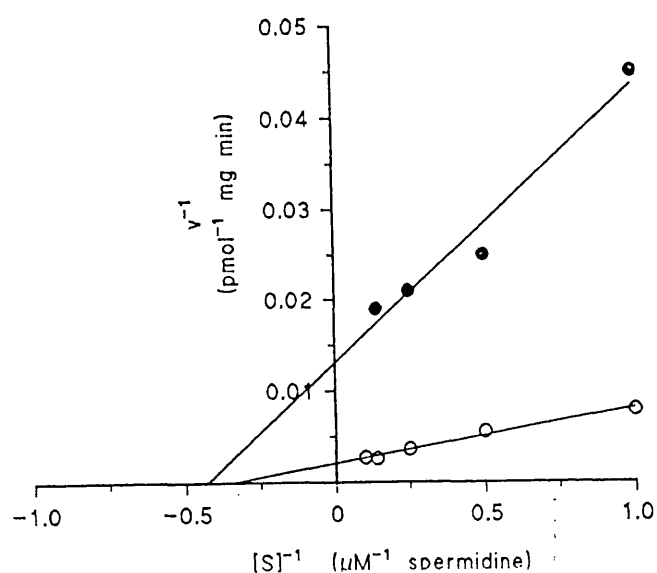
EATC (2.5×10^3) were incubated in the presence (O) or absence (●) of DFMO (0.5mM, 72 hr). The cells were then incubated with ^{14}C-spermidine (1.0 - 10.0 μM) for 30 min at 37°C. The rate of spermidine uptake is presented as both linear (a) and Lineweaver-Burk (b) plots. Data represent the mean of two experiments, each performed in duplicate.

3.2.2 The effect of MGBG on spermidine uptake and its *in vitro* toxicity to control and polyamine depleted EATC.

MGBG (50 and 100μM) competitively inhibited the uptake of ^{14}C-spermidine into EATC. The nature of this inhibition was classified as competitive since Lineweaver-Burk (reciprocal rate) plots of control and inhibited cells intercepted on the y axis (fig 3.5). The Ki value was was calculated from the following equation:-

$$\text{x-axis intercept} = \frac{-1}{Km \left(1+ \frac{[I]}{Ki} \right)}$$
(inhibited cells)

where [I] = concentration of inhibitor. The Ki value obtained for MGBG was *34.7 $\pm$ 4.3μM (n=5).

The *in vitro* toxicity of MGBG in control EATC and EATC depleted of polyamines by DFMO pretreatment (0.5mM, 72hr) was investigated. Cells were exposed to MGBG for 24hr followed by a further 48hr incubation with fresh medium. Viability was assessed by measuring ^{3}H-thymidine incorporation into DNA. The concentration of drug giving a 50% inhibition of radiolabel incorporation compared to control (no MGBG) cells (IC$_{50}$) was obtained. DFMO controls were used as appropriate. MGBG toxicity to EATC was increased approximately 10-fold by DFMO pretreatment (fig 3.6a) with IC$_{50}$ values of *10.8 $\pm$ 0.6μM (n=4) and *0.91 $\pm$ 0.24 μM (n=4) for control and DFMO pretreated EATC respectively.

A clonogenic assay (24hr exposure) was carried out to confirm the results obtained with ^{3}H-thymidine incorporation. The IC$_{50}$ obtained for MGBG was greater than 10μM in control cells and 1.0μM in DFMO pretreated cells (fig 3.6b). These results were comparable to those obtained in the ^{3}H-thymidine incorporation assay.

*mean $\pm$ s.e.m.

Fig 3.5   Kinetics of inhibition of spermidine uptake by MGBG in EATC.
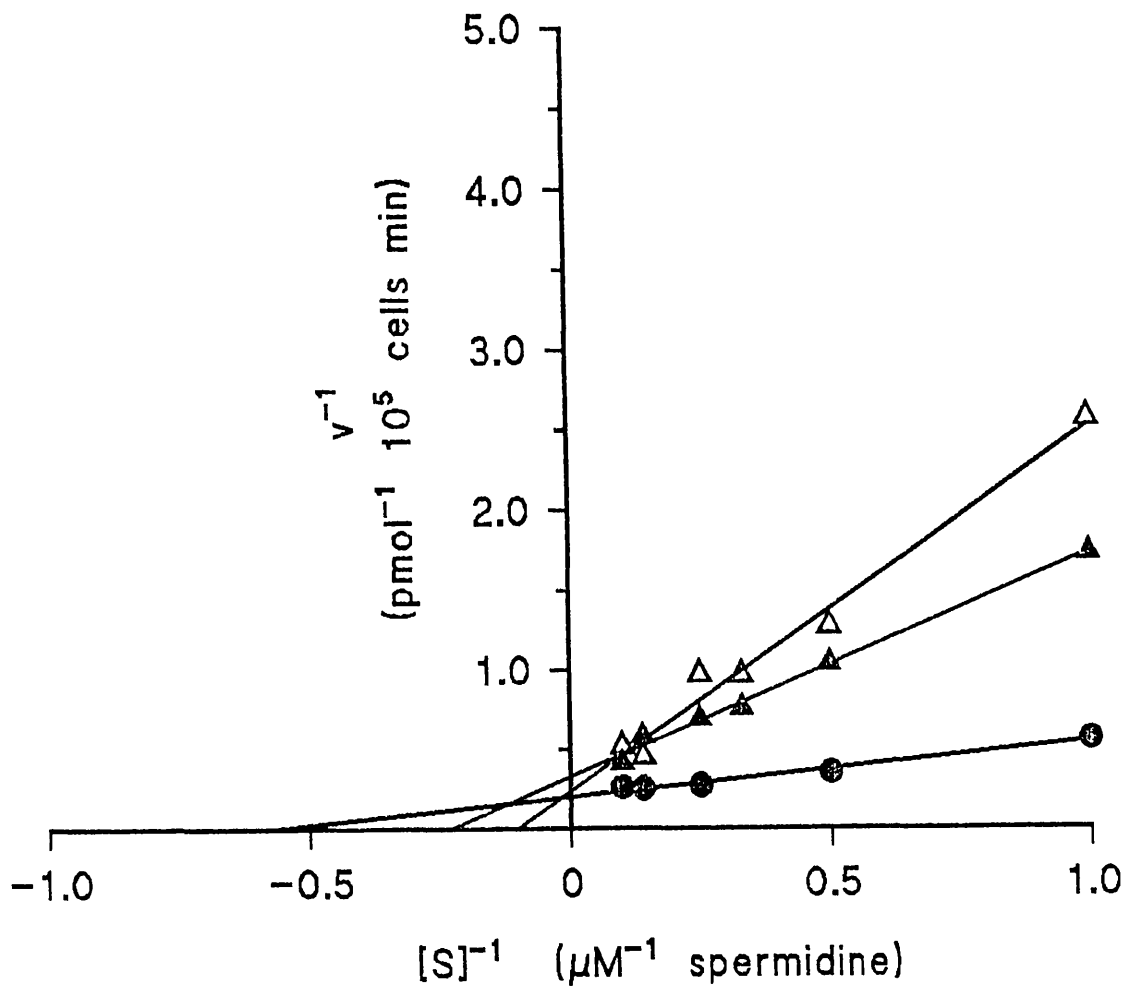
EATC (1x10^{5}) were incubated with ^{14}C-spermidine (0.5 - 10.0 μM) alone (●) or in the presence of MGBG (100μM ▵, or 50μM ▲) for 30 min at 37°C. The rate of uptake of labelled spermidine was measured and plotted as a Lineweaver-Burk plot using linear regression analysis and Ki values calculated. Data are representative of at least three experiments, each performed in duplicate.

Fig 3.6 Toxicity of MGBG to control and DFMO

pretreated EATC.

A
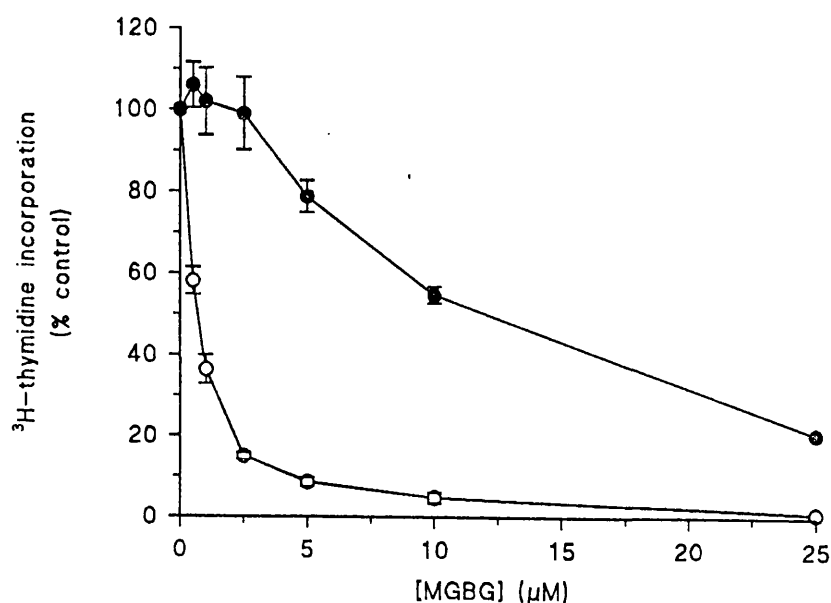
B
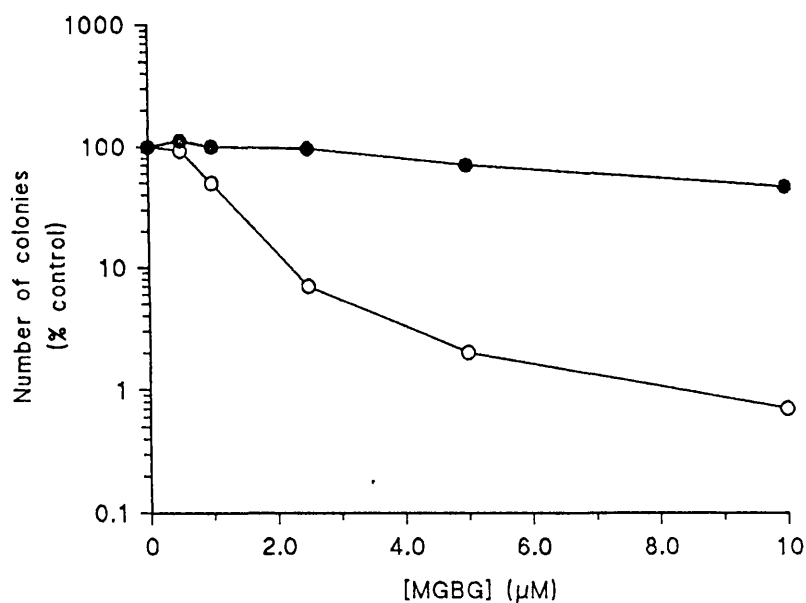
EATC were incubated in the presence (O) or absence (●) of
DFMO (0.5mM, 72 hr). MGBG (0 - 25 μM) was added for
24hr and cell viability measured using ^{3}H-thymidine
incorporation (a) and a clonogenic assay (b). Data for
(a) represent the mean ($\pm$ s.e.m.) of 4 experiments
performed in duplicate. Data for (b) are the results of
one experiment performed in triplicate.

In earlier experiments (data not shown) different MGBG exposure times were used in order to obtain the optimal exposure conditions for achieving toxicity. A 4hr exposure to MGBG resulted in much less toxicity whether this was measured immediately or after a post exposure incubation in fresh medium for 20 or 68 hr. Both 24 hr and 72 hr exposures to MGBG (no fresh medium added) resulted in a similar toxicity to that shown in fig 3.6.

A 24 hr exposure followed by a post exposure incubation in fresh medium for 48 hr was chosen as standard for future experiments since some drugs studied required a post exposure incubation period to allow DNA damage to be manifested as cell death.

3.2.3 The effect of a range of structurally related nitroimidazole-polyamine conjugates on spermidine uptake and their toxicity to EATC

Metronidazole and misonidazole were the non-conjugated control compounds for the 5- and 2-nitroimidazole series of compounds respectively. The ability of the nitroimidazole-polyamine conjugates (fig 3.1) to inhibit ^{14}C-spermidine uptake into EATC was measured as previously described (2.2.2). In all cases where examined kinetically the compounds inhibited spermidine uptake in a competitive manner. Ki values were obtained as described (3.2.2). The lower the Ki, the more potent the compound was as an inhibitor of uptake (table 3.1).

A greater than 700-fold variation was observed in the ability of the various compounds to inhibit the uptake of ^{14}C-spermidine, ranging from very potent inhibitors such as the 2-nitroimidazoles 430 and 230 (ki = 0.63 and 1.45μM respectively) and the triazole 530 (Ki = 0.66μM) to the non-conjugated parent compounds metronidazole and misonidazole which had no inhibition

Table 3.1 Inhibition of spermidine uptake in EATC by nitroimidazole-polyamine conjugates and their toxicity to control and polyamine depleted (DFMO) EATC.

Compound*	K_i (μM) ± s.e.m.	IC$_{50}$ (μM) Control	DFMO
Metronidazole	No inhibition	>100	>100
121	174 ± 49.8 (4)	80	15 (3)
131Me	184 ± 70 (3)	>100	71 (2)
111	400 (1)	>100	>100 (1)
Misonidzole	No inhibition	>100	>100 (2)
220	40 ± 7.1 (3)	88.5	25.5 (2)
420	329 (2)	>100	60 (2)
430Me	19.3 ± 2.65 (3)	>100	>100 (2)
430	0.63 ± 0.14 (3)	>100	>100 (2)
230Me	4.66 ± 1.50 (3)	>100	93 (2)
530	0.66 (2)	>100	>100 (2)
230	1.45 (2)	42.7	9.0 (2)
MGBG	29.8 ± 7.9 (4)	12.7 ± 4.2 (4)	0.8 ± 0.14 (4)

EATC (1×10^5) were incubated at 37°C for 30 min with ^{14}C-spermidine (1-10μM) either in the absence of presence of the compound under study (1, 10, 50 or 100μM). The accumulation of ^{14}C-label into the cells was measured, the Ki values determined from Lineweaver-Burk plots and the mean values shown.

EATC (2.5×10^3) were also incubated in the presence or absence of DFMO (0.5mM) for 72 hr. The compound under study (0-100μM) was added for 24 hr, the medium replaced and the cells incubated for a further 48 hr. ^{3}H-Thymidine was added for the final 2 hr of the incubation and incorporation into DNA measured. The concentration of compound inhibiting ^{3}H-thymidine incorporation by 50% (IC$_{50}$) was determined.
* Structures of compounds are given in fig 3.1.

within the range of concentrations tested (0-100μM). 2-Nitroimidazole conjugates were, in general, better inhibitors of spermidine uptake than 5-nitroimidazole conjugates (table 3.1).

The *in vitro* toxicity of the nitroimidazoles and their polyamine conjugates was assessed by their ability to inhibit ^{3}H-thymidine incorporation into EATC. IC_{50} values obtained are shown in table 3.1. The lower the IC_{50} value the more toxic the compound. Neither metronidazole nor misonidazole were toxic to control or DFMO pretreated EATC (table 3.1). However a number of the conjugates exhibited some toxicity and for several, namely 121, 220 and 230, toxicity was markedly enhanced by polyamine depletion. A clonogenic assay (24hr exposure) with compound 230 confirmed the results for this compound with IC_{50} values of 40μM and 20μM obtained for control and DFMO pretreated cells respectively (data not shown).

Compound 430, a particularly potent inhibitor of spermidine uptake, was studied further. In order to investigate the effect of the nitroimidazole group on the inhibitory activity of 430, 2 structural analogues, P(I) (N^1,N^8-bis(2-hydroxy-2-phenyl-ethyl)-spermidine) and P(II) (N^1,N^8-bis(2-hydroxy-3-phenylether-propyl)-spermidine) (fig 3.2), with identical polyamine backbones to compound 430, but lacking the nitroimidazole groups, were studied. Their ability to inhibit ^{14}C-spermidine uptake was compared to compound 430. Both were found to be competitive inhibitors of uptake (fig 3.7) although their potency was substantially less than that for compound 430. (Ki (P(I)) = 17.8μM, Ki (P(II)) = 7.7μM).

A further experiment to determine whether the inhibition of spermidine uptake by 430 was reversible was performed by preincubating cells with 430 prior to measuring ^{14}C-spermidine uptake. No difference in uptake was observed for control and preincubated cells (fig 3.8). The effect of compound 430 on the toxicity of MGBG

Fig 3.7 Kinetics of inhibition of spermidine uptake by compound 430 and two structural analogues in EATC.
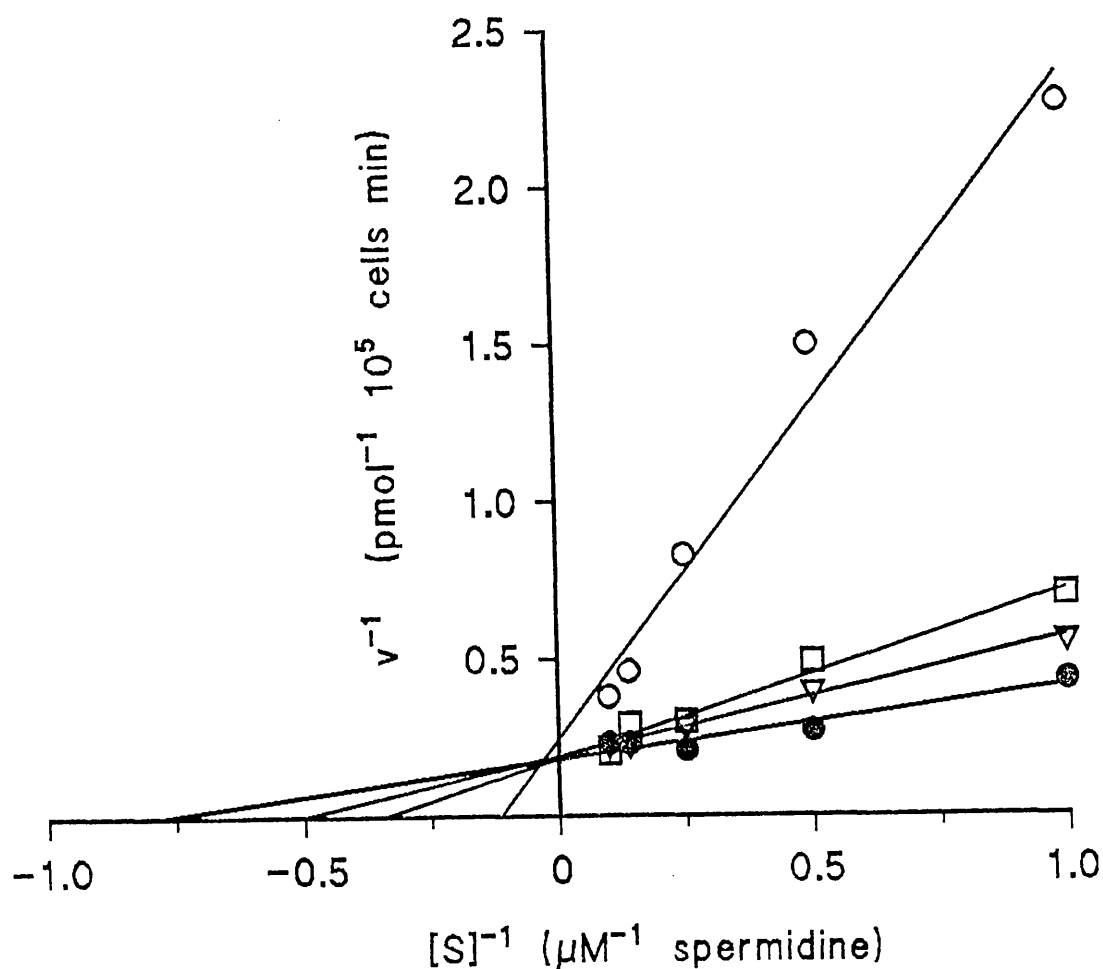
EATC (1×10^5) were incubated for 30 min at 37°C with ^{14}C-spermidine alone (●) or in the presence of compound 430 (1μM)(o), analogue P(I) (10μM)(▽) or analogue P(II) (10μM)(□). The rate of uptake of ^{14}C-spermidine was measured and plotted as a Lineweaver-Burk plot from which Ki values were calculated. Data are representative of 2 experiments performed in duplicate.

Fig 3.8 The effect of preincubation of EATC with compound 430 on spermidine uptake.
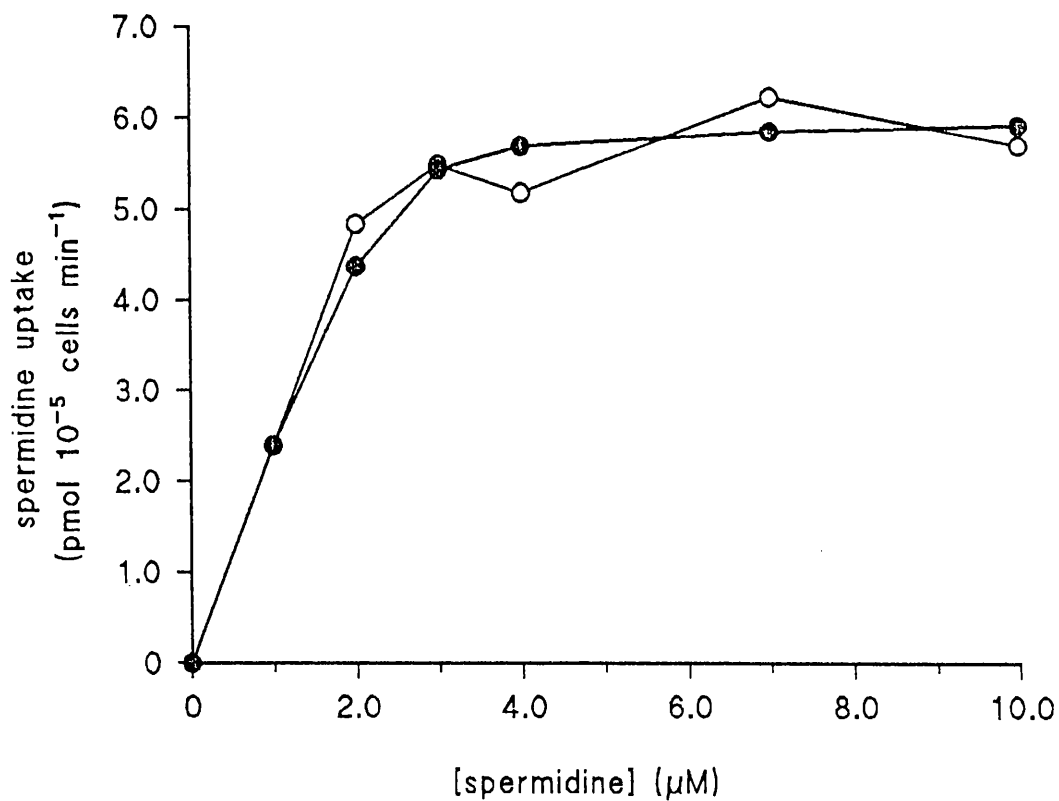
EATC (1×10^5) were incubated in the presence or absence of compound 430 for 4 hr and medium replaced. ^{14}C-spermidine uptake by control (●) and 430 pre-incubated (○) cells was measured for 30 min at 37°C. The data presented are the results of one experiment.

to polyamine depleted cells by coincubation of the cells
with both drugs was also investigated. A concentration
dependent inhibition of MGBG toxicity by compound 430 was
seen (fig 3.9). Spermidine coincubation (10μM) with MGBG
also inhibited MGBG toxicity (fig 3.9). In addition, an
experiment was performed to determine whether compound 430
could replenish polyamine levels in polyamine depleted
cells in a similar manner to spermidine. Compound 430
(10μM) or spermidine (10μM) were added to polyamine
depleted EATC for 4 hr followed by washing and the
addition of fresh medium. MGBG toxicity was assessed.
Spermidine preincubation gave a marked reduction in MGBG
toxicity whereas compound 430 had no effect (fig 3.10).

3.2.4 Inhibition of spermidine uptake by a range of polyamine analogues

The abilities of a range of putrescine and
spermidine analogues, provided by Prof. B. Golding (Univ.
of Newcastle) to inhibit ^{14}C-spermidine uptake was
investigated (table 3.2). The effect of different
substituents, present on the polyamine backbone, on the
inhibition of spermidine uptake were reflected in the Ki
values obtained (table 3.2).

Fig 3.9 Effect of compound 430 on MGBG toxicity to DFMO pretreated EATC.
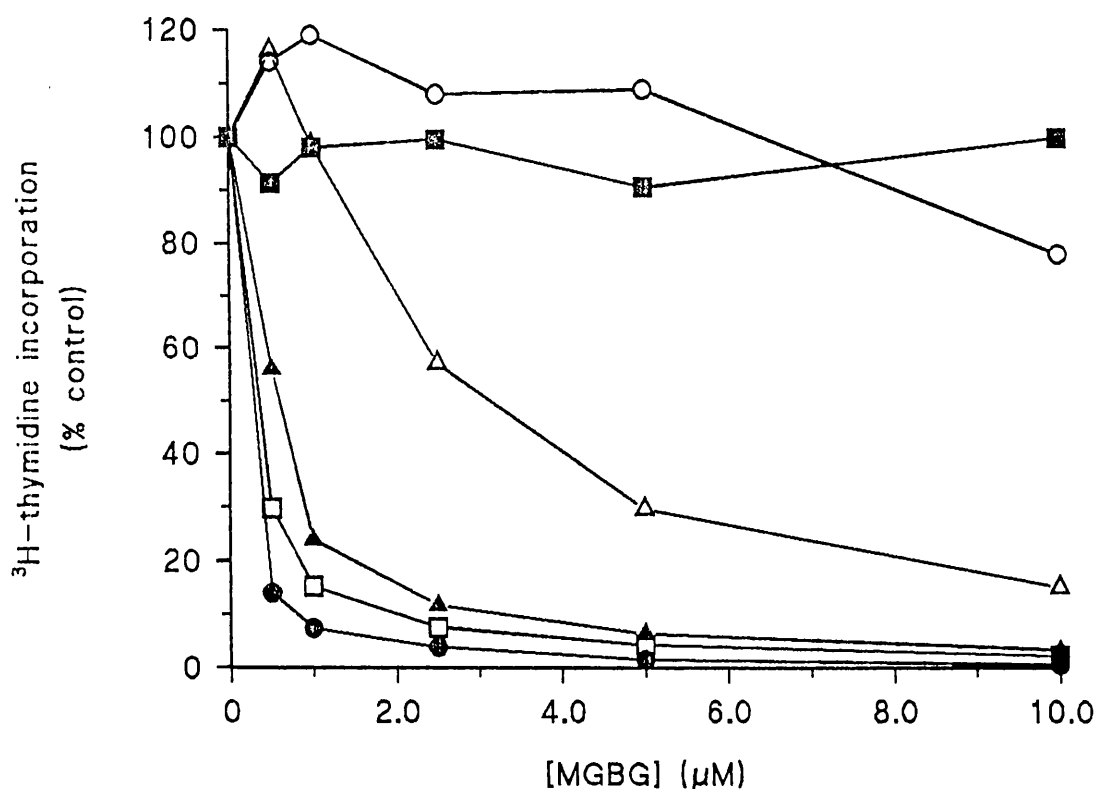
EATC (2.5×10^3) were incubated with DFMO (0.5mM) for 72 hr. MGBG (0-10μM) was added to cells alone (●) or with spermidine (10μM,■) or compound 430 (0.05μM,□), (0.1μM,▲), (1μM,△) and (10μM,○) for 24 hr. Medium was replaced and the cells incubated for a further 48 hr. ^{3}H-Thymidine was added for the final 2 hr and the amount of label incorporated into DNA measured and expressed as a percentage of relevant control cells. The data presented are the results of one experiment.

Fig 3.10 Effect of spermidine and compound 430

preincubation on MGBG toxicity to DFMO pretreated EATC.
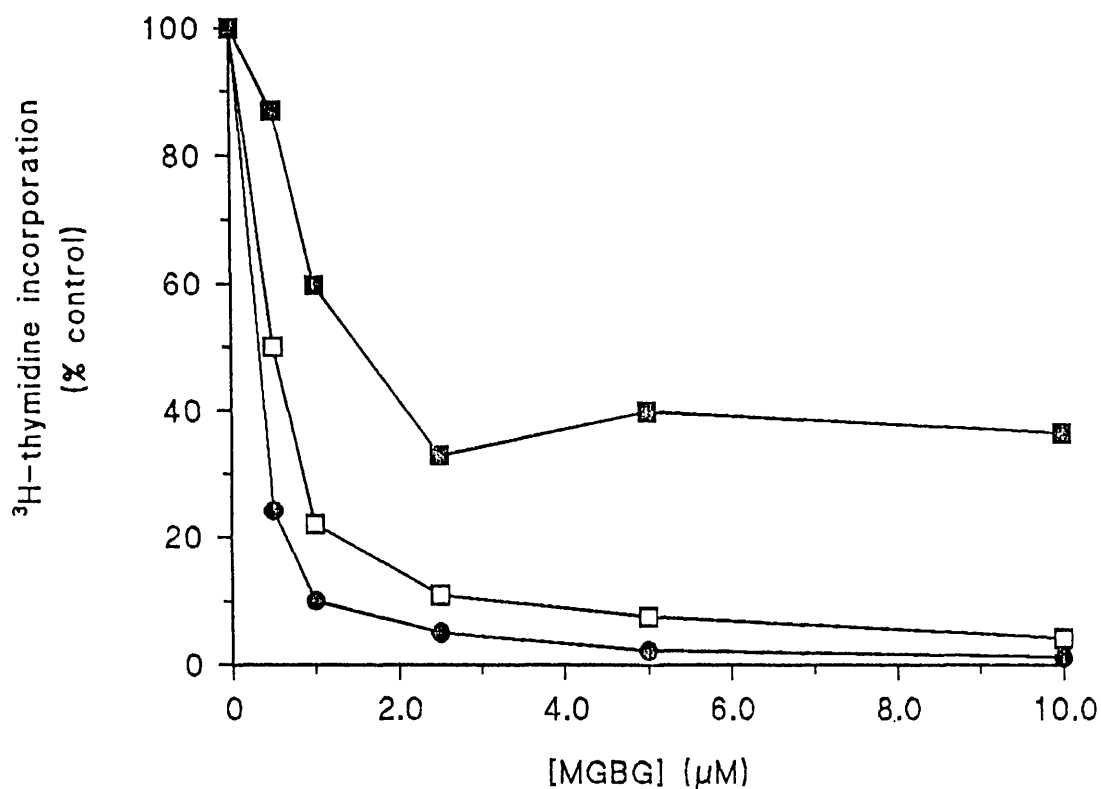
EATC (2.5×10^3) were incubated with DFMO (0.5mM) for 72 hr.
Spermidine (10μM) or compound 430 (10μM) were added to
the cells for 4 hr and the medium replaced. MGBG (0 - 10
μM) was added to the cells for 24 hr followed by the
addition of fresh medium for a further 48hr. The
toxicity of MGBG to cells pretreated with DFMO alone (●)
was compared to those pretreated with DFMO+SPD (■) and
DFMO+430 (□) using ^{3}H-thymidine incorporation to measure
cell viability. The data presented are the results of
one experiment.

TABLE 3.2   Inhibition of spermidine uptake by
putrescine and spermine analogues

Compound	Spermidine uptake Ki (μM)
$H_2N(CH_2)_4NH(CH_2)_2OH$	28.0
$H_2N(CH_2)_4NHCH_3$	27.5
$CH_3NH(CH_2)_4N(CH_2)_3$	87.9
$(CH_3)_2N(CH_2)_4N(CH_3)_2$	348
$CH_3CONH(CH_2)_4NH(CH_2)_4NH_2$	257
$NH_2(CH_2)_4NH_2$	65
$(Me)_2N(CH_2)_3NH(CH_2)_4N(Me)_2$	31.9
$H_2N(CH_2)_3NMe(CH_2)_3NH(CH_2)_2SH$	31.9
$(Me)_3N^+(CH_2)_3N^+(Me)_2(CH_2)_3N^+(Me)_3$ .3HCl	No inhibition
MGBG	19.7

EATC (1×10^5) were incubated at 37°C for 30 min with ^{14}C-spermidine (1-10μM) alone or with the compound under study (1, 10, 50 or 100μM). The accumulation of ^{14}C-label into the cells was measured, the Ki values determined from Lineweaver-Burk plots and the mean values shown.

3.2.5 The effect of CLB-SPD conjugate and chlorambucil on spermidine uptake and their toxicity to control and DFMO pretreated EATC.

The structure of the CLB-SPD conjugate used in these studies is shown in fig 3.2. CLB-SPD competitively inhibited ^{14}C-spermidine uptake into EATC (fig 3.11) (Ki = 13.3 ± 1.0μM (n=5))*. Chlorambucil, the non-conjugated parent compound, did not effect spermidine uptake (data not shown).

CLB-SPD toxicity (assessed by inhibition of ^{3}H-thymidine incorporation) was increased 5-fold by polyamine depletion (fig 3.12) resulting in an IC_{50} value of 13.1 ± 4.3μM* (n=3) for polyamine depleted EATC compared to 70.8 ± 10.4 μM* (n=3) for control EATC. This DFMO effect was confirmed in a clonogenic assay (fig 3.13) (IC_{50}(control) = 15μM*, IC_{50}(DFMO) = 3.5μM)

Chlorambucil was more toxic than CLB-SPD to control EATC (fig 3.12). Its toxicity was slightly increased by DFMO pretreatment (fig 3.12) (IC_{50}(control) = 16.3 ± 5.6μM* (3), IC_{50}(DFMO) = 9.2 ± 0.88μM*). These results were confirmed using a clonogenic assay (data not shown).

3.2.6 Comparison of the *in vitro* toxicity of CLB-SPD and chlorambucil to L1210 tumour cells

The toxicity of CLB-SPD and chlorambucil to L1210 cells *in vitro* was investigated. CLB-SPD (IC_{50}= 0.33μM) was approximately 5-fold more toxic than chlorambucil (IC_{50}= 1.75μM) following a 24hr exposure of cells to the drugs when inhibition of ^{3}H-thymidine incorporation into DNA was used to measure toxicity (fig 3.14)

* mean ± s.e.m.

Fig 3.11 Inhibition of spermidine uptake in EATC by CLB-SPD.
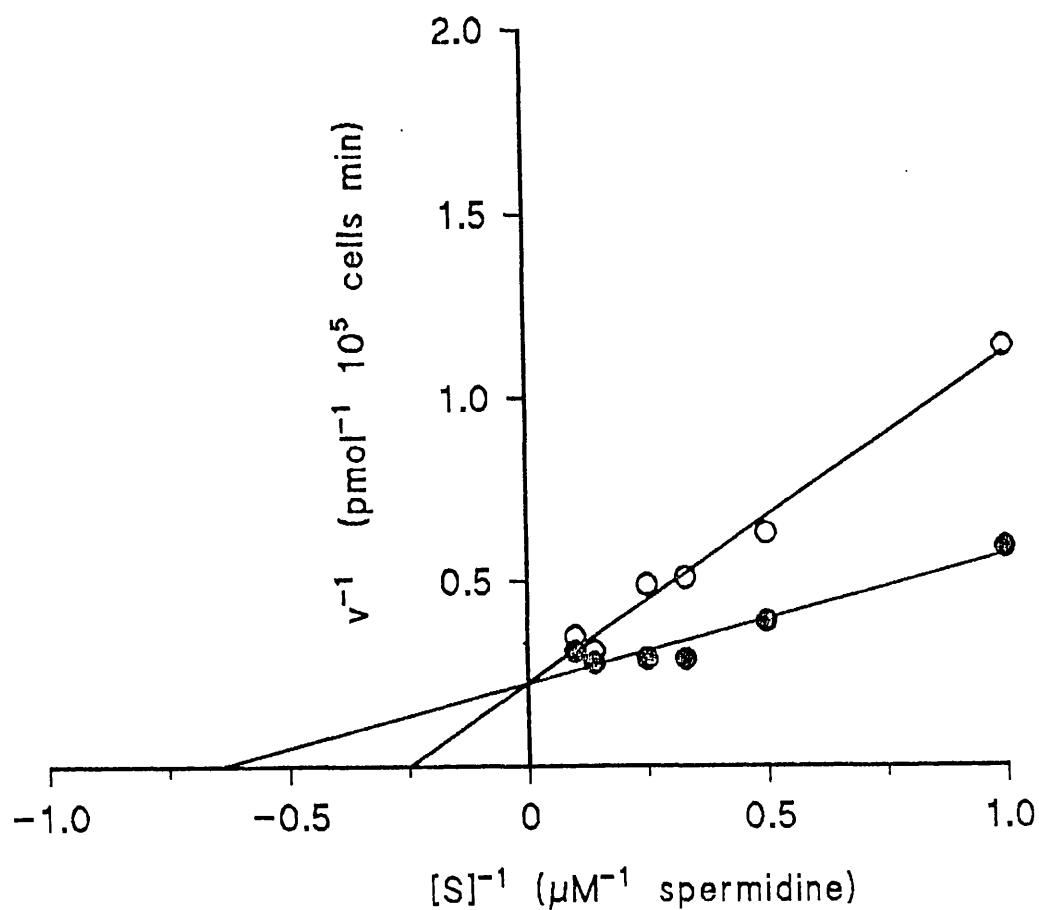
EATC (1x10^5) were incubated for 30 min at 37°C with ^{14}C-spermidine (1 - 10 μM) alone (●) or in the presence of CLB-SPD (25μM) (O). Results are presented as a Lineweaver-Burk plot. Data are representative of at least two experiments performed in duplicate.

Fig 3.12 Toxicity of CLB-SPD (a) and chlorambucil (b) to control and DFMO pretreated EATC (^{3}H-thymidine incorporation).

A

B
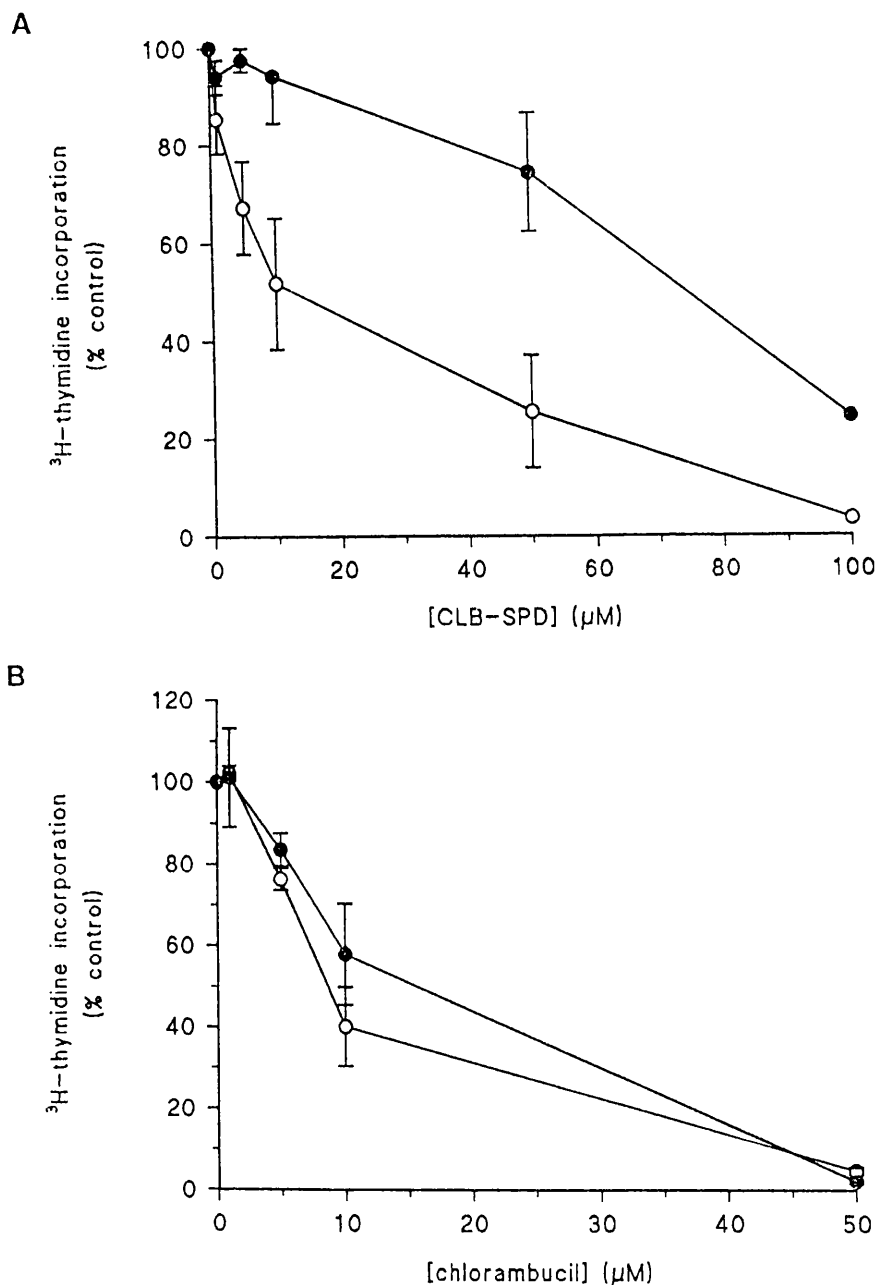
EATC (2.5 x 10^3) were incubated for 72 hr in the presence (O) or absence (●) of DFMO (0.5mM). CLB-SPD (a) or chlorambucil (b) (0 - 100 μM) were added for 24 hr and fresh medium added for a further 48 hr. ^{3}H-thymidine was added for the final 2 hr of the incubation and the amount of label incorporated into DNA measured. Data represent the mean (± s.e.m.) of at least 3 experiments performed in duplicate.

Fig 3.13   Toxicity of CLB-SPD to control and DFMO

pretreated EATC (clonogenic assay).
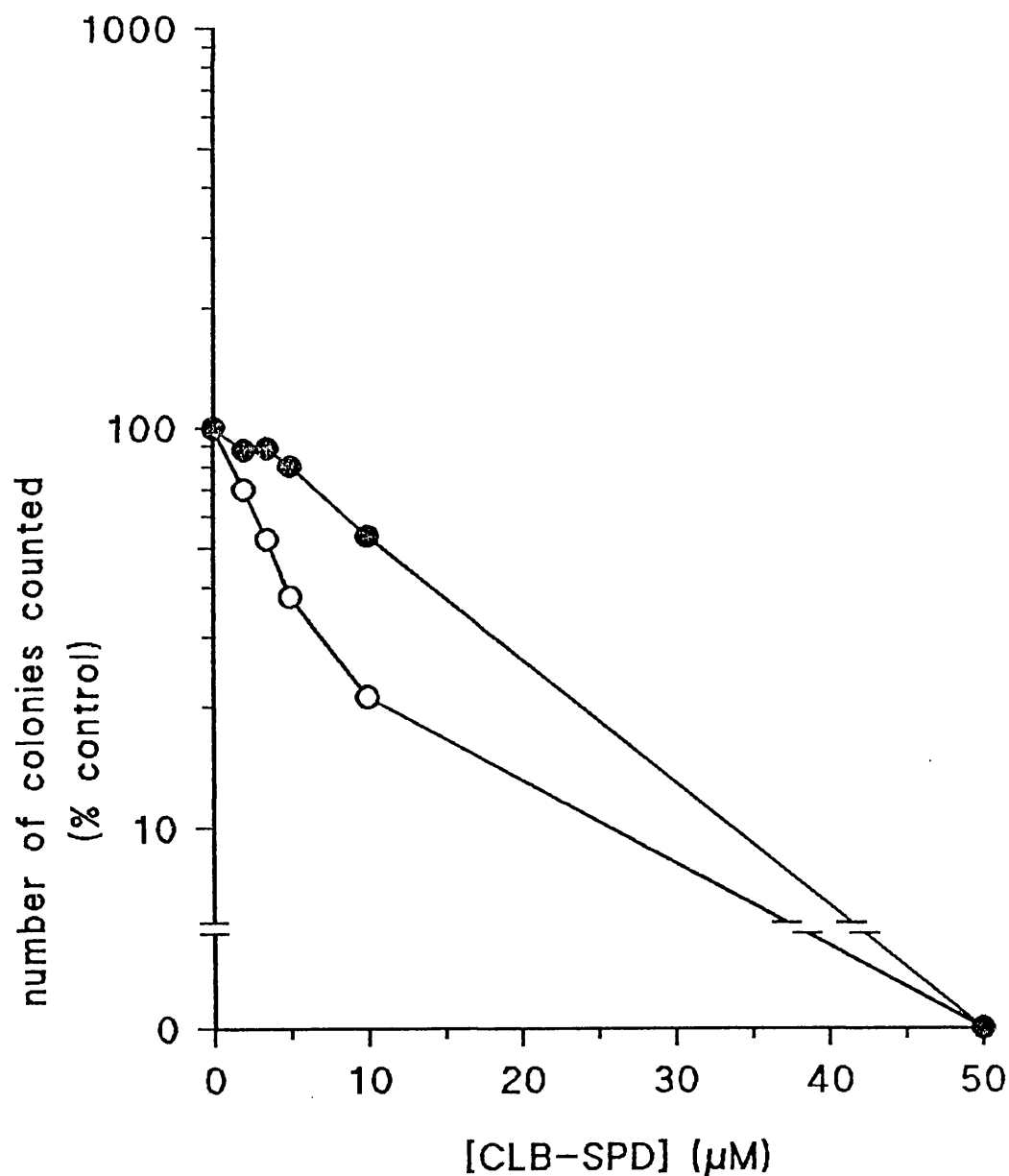
Control (●) and DFMO (O) (0.5mM, 72 hr) pretreated cells
were exposed to CLB-SPD (0 - 50 μM) for 24hr and
following trypsinization, seeded at different densities
in petri dishes. The number of colonies present after 2
weeks were counted and compared to control or DFMO
pretreated cells (no CLB-SPD).

Fig 3.14 The toxicity of CLB-SPD and chlorambucil to L1210 cells.
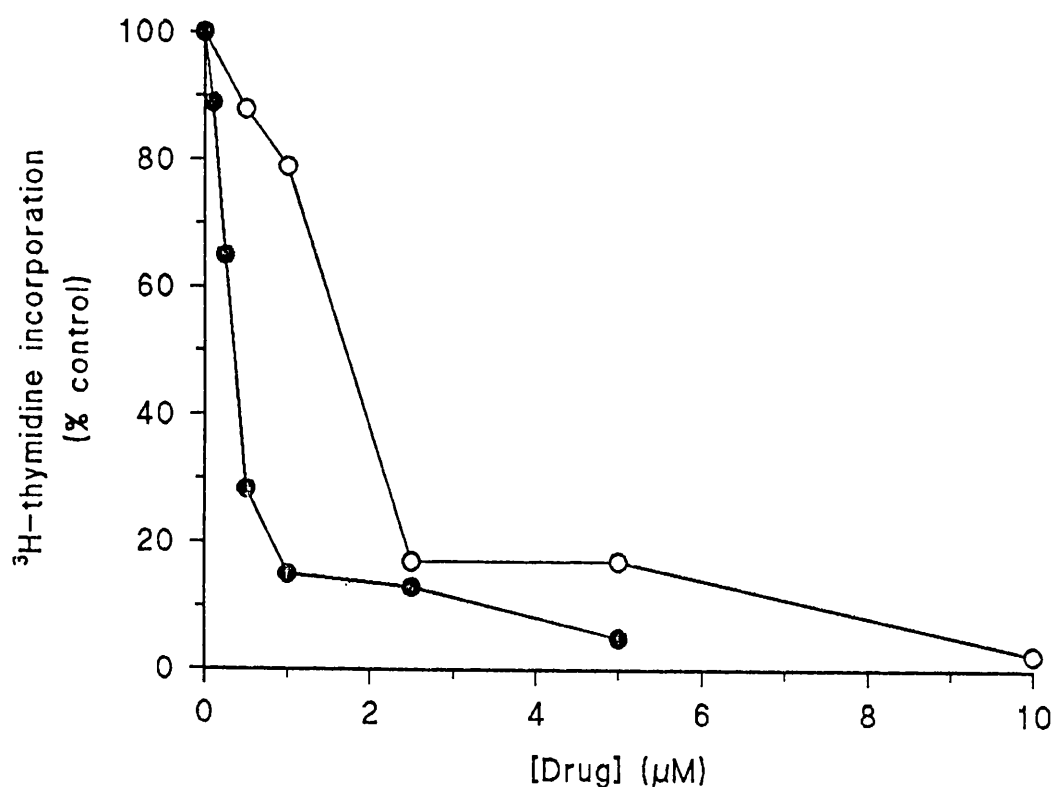
L1210 cells (1x10^5/ml) were exposed to CLB-SPD (●) or chlorambucil (○) (0 - 10μM) for 24 hr and fresh medium added for a further 48hr. Toxicity was assessed using the ³H-thymidine incorporation assay. Data represent the means of two experiments performed in duplicate.

3.2.7 *In vivo* antitumour studies with EATC and L1210 cells.

The *in vivo* antitumour activity of CLB-SPD, chlorambucil, MGBG and compound 230 against EATC was investigated. In an initial experiment (EXP 1, table 3.3), single equimolar doses of CLB-SPD (5mg/kg) or chlorambucil (2.4 mg/kg) (i.p.) gave small but similar increases in life-span compared to their respective vehicle control groups (table 3.3). DFMO pretreatment of animals had no effect on the antitumour activity of either compound (data not shown).

In a second experiment, it was attempted to administer a larger dose of CLB-SPD in order to obtain a better antitumour effect. However, a lethal acute CNS toxicity was observed with doses greater than 15mg/kg. Mice rapidly developed effort tremors and an unsteady gait within 3-4 min of CLB-SPD administration, progressing to prostration at 5-6 min. At 15 min clear coarse whole body effort tremors were observed and the mice were unwilling or unable to move. Fatalities were observed from 8-170 min. The LD_{50} was 17.5mg/kg for a single i.p. dose (results not shown). CNS toxicity was evident for chlorambucil, but at 10-fold higher doses, and did not cause death (results not shown).

Multiple doses of CLB-SPD (eg 2 x 10mg/kg and 2 x 12.5mg/kg on days 1 and 3) were tolerated and therefore used in antitumour studies. Multiple i.p. doses of CLB-SPD (2 x 10mg/kg and 2 x 12.5 mg/kg) resulted in a 26 % and 35 % increase in life-span respectively (Table 3.3, EXP 2). Chlorambucil (2 x 5 mg/kg) gave a larger increase in life span than CLB-SPD with four out of five animals surviving for longer than 30 days (table 3.3, EXP 2). The nitroimidazole-polyamine conjugate 230 which demonstrated some *in vitro* activity against EATC, also gave a small increase (+25%) in life-span of EATC bearing mice (table 3.3, EXP 2). MGBG (2x40mg/kg) did not give an increase in life span in this study (table 3.3).

Table 3.3 *In vivo* antitumour activity of various compounds against EATC.

Experi-ment	treatment	life-span (days ± SD)	% increase in life-span
	Vehicle (10% PG in 0.9% NaCl)	13.6 ± 0.5	–
	chlorambucil (2.4 mg/kg)	14.6 ± 0.5	+8.5%
EXP 1	chlorambucil (20mg/kg)	4/5 alive day 30	> +120%
	Vehicle (DMEM)	16.0 ± 2.2	–
	CLB-SPD (5mg/kg)	18.0 ± 0	+12.5%
	Vehicle x2 (10% PG in 0.9% NaCl)	14.2 ± 1.8	–
	chlorambucil (5mg/kg x 2)	4 alive day 30	> +110%
	Vehicle x 2 (KRP)	14.8 ± 2.95	–
	CLB-SPD (10 mg/kg x 2)	18.75 ± 0.5	+26.7%
EXP 2	CLB-SPD (12.5 mg/kg x 2)	19.0 ± 0.5	+35.5%
	MGBG (40 mg/kg x 2)	15.0 ± 2.2	+1.4%
	Vehicle x 3 (KRP)	13.5 ± 0.5	–
	Compound 230 (10mg/kg x 3)	19.6 ± 1.7	+25%
	Vehicle (10% PG in 0.9% NaCl)	15.7 ± 1.86	–
	chlorambucil (10mg/kg)	19.9 ± 1.86	+24.8%
EXP 3	chlorambucil (10mg/kg) + spermidine (4 mg/kg)	20.8 ± 3.2	+32.5
	chlorambucil (15mg/kg)	23.2 ± 1.8	+47.8

KEY PG=propylene glycol   KRP=Kreb's Ringer phosphate

Balb/c mice were innoculated i.p with EATC (1×10^6) on day 0 of the study. Mice were dosed i.p. with test compound or vehicle on day 1 (experiments 1 and 3) or on days 1 and 3 or days 1,3 and 5 (experiment 2). The time taken for the animals to die or become moribund as the result of ascites growth was recorded.

In a third experiment, single i.p. doses of chlorambucil gave a dose dependent increase in survival time (table 3.3, EXP 3). Co-administration of spermidine (4mg/kg) with chlorambucil (10 mg/kg) did not effect the *in vivo* antitumour activity of chlorambucil (table 3.3).

In mice innoculated with L1210 tumour cells neither CLB-SPD (10mg/kg) nor chlorambucil (20mg/kg) gave an increase in survival of mice innoculated with the L1210 tumour (table 3.4). In contrast, the antimetabolite 5-fluorouracil (20mg/kg) and MGBG (50mg/kg) did give increases in life-span (table 3.4).

Table 3.4 *In vivo* antitumour activity of various compounds against L1210 cells.

Experi-ment	treatment	life-span (days ± SD)	% increase in life-span
	Vehicle (10% PG in 0.9% NaCl)	19.5 ± 5.0	–
	chlorambucil (20 mg/kg)	17.2 ± 0.4	-12%
EXP 1	Vehicle (KRP)	21.8 ± 3.5	–
	CLB-SPD (10mg/kg)	21.35 ± 3.5	-2%
	5-fluorouracil (20mg/kg)	4 alive day 30	> +40%
	MGBG (50mg/kg)	4 alive day 30	> +40%

KEY PG=propylene glycol KRP=Kreb's Ringer phosphate

C57/DBA2 mice were innoculated i.p. with L1210 cells (1x 10^5) on day 0 of the study. On day 1, mice were administered a single i.p injection of test compound or vehicle. The time taken for the mice to die or become moribund due to ascites growth was recorded.

111

3.3 Discussion

The initial kinetic studies on EATC confirmed results previously reported for these cells (78). In this study EATC had a saturable, concentration and temperature dependent uptake system for spermidine with $K_m = 2.2\mu M$, compared to $3\mu M$ reported by Seppanen et al (78). Vmax values were however more than 10-fold greater in this study ($6.2pmoles/10^5$ cells/min) than in Seppanen's ($0.47pmoles/10^5$ cells/min). This difference may be due to the fact that Seppanen's observations were made in EATC which had been adapted for growth in suspension culture whereas in this study the EATC were grown as monolayers.

As previously reported in the literature (72,78,153), pretreatment of EATC with DFMO resulted in a 6-fold increase in the maximal velocity of spermidine uptake (fig 3.4). No change in K_m was observed suggesting that increased uptake was probably due to an increase in the number of receptors within the cell rather than to an increased number of cell surface receptors. This is also reported by Seppanen et al. (78). Since no increase in amino acid transport is seen (72) it is believed that the increased uptake stimulated by DFMO pretreatment is specific for polyamines.

MGBG was investigated for its suitability for use as a positive control in uptake competition studies and in vitro toxicity studies. MGBG competitively inhibited spermidine uptake in EATC in a similar manner to that observed in L1210 cells (120,148). In addition, the toxicity of MGBG was increased by polyamine depletion (fig 3.6), an observation also widely reported in the literature (72,147,153). This, together with the observation that the uptake of MGBG is increased in polyamine depleted EATC (72,78,147,153), is indicative that MGBG is a substrate of the polyamine uptake system. A further point is that, in this study, the toxic effects of MGBG were not detectable by ^3H-thymidine incorporation

until 24 hr after the initial addition of the drug. This was observed by Seppanen et al (153) who report that, with MGBG, changes in protein synthesis are detectable prior to any DNA effects.

The addition of exogenous spermidine (10μM) to polyamine depleted EATC is reported to replenish polyamine levels within 2 hours (72) which explains why MGBG toxicity to DFMO pretreated EATC in this study was partially reversed by the addition of spermidine to the cells for 4 hours (fig 3.10) prior to MGBG exposure.

The results of these studies demonstrated that MGBG was suitable for use as a positive control compound. The observation that ^{3}H-thymidine incorporation correlated well with the clonogenic assay (fig 3.6) for assessing cytotoxicity also demonstrated that ^{3}H-thymidine incorporation was suitable for screening the novel compounds for *in vitro* toxicity.

Owing to the lack of a simple assay for the nitroimidazoles or their polyamine conjugates, their ability to competitively inhibit spermidine uptake into EATC was used to suggest that they may also be taken up into the cells by either the same or very similar carrier systems. Support for this comes from previous studies from a number of laboratories (56,60,70). However, recent studies using B16 melanoma cells (80) have shown that although paraquat inhibits competitively the uptake of putrescine, neither putrescine, spermidine nor spermidine inhibit paraquat uptake suggesting that either more than one polyamine uptake system exists or that paraquat and the polyamines are not taken up by the same system. Thus, whilst studies demonstrating the competitive inhibition of polyamine uptake into cells suggest uptake of the inhibitors, they are not definitive.

In this study, the ability of a selection of nitroimidazole-polyamine conjugates and their non-conjugated parent compounds to competitively inhibit

spermidine uptake into EATC was investigated. Low Ki values were indicative of good inhibitors of spermidine uptake. None of the 5-nitroimidazole compounds were particularly good inhibitors of spermidine uptake (table 3.1). Both 121 (a diamine) and 131Me (a triamine) were poor inhibitors although better than the monoamine 111. This was due possibly to the fact that only three carbon atoms separated the nitrogen atoms in both the diamine and triamine, and a separation of at least four carbon atoms is required for a compound to be a good inhibitor of the polyamine uptake system (77,125,126,127). Monoamines are also poor inhibitors of uptake (126).

The 2-nitroimidazole-polyamine conjugates were more effective inhibitors of spermidine uptake than the 5-nitroimidazole conjugates (table 3.1) even though, in a number of these compounds, the two nitrogen atoms of the polyamine moiety were separated by only three carbon atoms. This may be related to the nature of the linkage of the polyamine to the nitroimidazole . In the 5-nitroimidazoles, the polyamine is joined to the C^2 of the nitroimidazole ring via a sulphur linkage whereas the 2-nitroimidazole compounds are conjugated to the polyamine via the N^1 of the imidazole ring, without a sulphur linkage. Thus in the 2-nitroimidazole conjugates there is effectively one additional nitrogen forming part of the polyamine chain. Additionally, this nitrogen atom may be more positively charged due to the electron withdrawing potential of the nitro group attached to the adjacent carbon atom on the ring. The triazole 530, which was also a good inhibitor of spermidine uptake (table 3.1), also shares this structural characteristic. A further point to consider is the greater electron affinity of 2-nitroimidazole compared to the 5-nitroimidazole (284) which could also affect the charge distribution on the polyamine chain.

Two of the best inhibitors of spermidine uptake were compounds 430 (Ki =0.63μM) and 230 (Ki =1.45μM).

Both these compounds contain a similar and possible optimal spacing between the nitrogen atoms of the polyamine moiety to that found in spermidine. Compound 230 is also an N^4 substituted spermidine. The N^4 position is reported to be the optimal position for substitution in order to retain affinity for the uptake system (97,122). It is reported that even N^4-benzyl substituted spermidine is a good inhibitor of uptake (97,122). In addition to being a good inhibitor of spermidine uptake, compound 230 was also cytotoxic. Cytotoxicity was enhanced by polyamine depletion with DFMO.

In contrast to 430 and 230, compounds 430Me and 230Me were less effective inhibitors of spermidine uptake. However, the nitrogens of the polyamine backbones in these compounds were separated by only three carbon atoms. The differences in the inhibitory potency may have been due to the differences in spacing of the nitrogen atoms of the polyamine. In addition, the presence of the methyl group on the central nitrogen of these compounds may have also have reduced the cationic nature of the nitrogen atom and reduced the inhibitory activity of the compounds. This may also partially explain the poor inhibitory potency of the 5-nitroimidazole conjugate 131Me, which also has a methyl group on the central nitrogen.

Some preliminary studies were carried out on the effect of the nitroimidazole moieties on the ability of the conjugates to inhibit spermidine uptake. Substitution of the two nitroimidazole groups of compound 430 (the most effective inhibitor of uptake) with alkyl phenyl substituents (ie. non N-containing groups)(P(I) and P(II))(fig 3.2) resulted in up to a 30-fold decrease in the inhibitory potency (fig 3.7). These results provide further evidence in favour of the suggestion that the extra (positively charged?) nitrogen of the nitroimidazole group enhances the compound's potency at

inhibiting spermidine uptake. This is similar to the observation that increasing the number of positive charges in a series of pyridinium quarternary salts increased their ability to inhibit polyamine uptake (125).

Compound 430 was further investigated with regards to its ability to inhibit spermidine uptake. Its inhibitory effects on spermidine uptake were found to be reversible in that spermidine uptake was unaffected by preincubation of EATC with the compound (fig 3.8). This suggests that the compound does not become permanently associated with the receptor on the cell surface but can be washed off. In addition compound 430 (itself non-toxic), like spermidine, was able to inhibit MGBG toxicity to DFMO pretreated EATC in a concentration dependent manner (fig 3.9). This is probably due to competition for uptake. However, the observation that, unlike spermidine (fig 3.10), preincubation of polyamine depleted EATC with compound 430 prior to MGBG exposure did not inhibit the DFMO effect, suggests that 430 does not replenish polyamine levels and therefore does not function as an active polyamine.

The *in vitro* toxicity of the nitroimidazole-polyamine conjugates to control and polyamine depleted EATC was investigated (table 3.1). The parent nitroimidazole compounds exhibited no toxicity to EATC whereas a number of the conjugates did have moderate toxicity, especially following polyamine depletion (namely 121, 220 and 230). There was no direct relationship between the toxicity of the conjugates and their ability to inhibit spermidine uptake. For example, for some compounds (eg. 121 and 220), considerable toxicity to polyamine depleted cells was shown, although they were poor inhibitors of spermidine uptake (table 3.1) This suggests that significant uptake of these compounds actually occurred, especially into polyamine depleted cells. This is possible since the uptake

studies were carried out over a 30-min time period whereas the toxicity studies involved a 24 hr exposure, which may have allowed accumulation of the compound despite its low affinity for the uptake system. In addition, it has been recognized that there is more than one uptake system for polyamines (56,82,85,102,104). Compounds 121 and 230, which had a diamine backbone, may have entered the cells via an uptake system for diamines rather than utilizing an uptake system for spermidine.

Polyamine depletion brings about conformational changes in DNA, reducing its stability and altering its susceptibility to DNA damaging agents (160,161,162, 165,286). It also leads to an accumulation of EATC in the G2 and S phases of the cell cycle (Pegg 1982). It is possible that the nitroimidazole compounds may exhibit some cell cycle specificity and polyamine depletion rendered the cells more susceptible to their toxicity rather than increasing their uptake. However, if this was the case, one would also have expected the parent compounds to have an enhanced toxicity in polyamine depleted cells, which did not occur. Polyamine depletion, however, may increase the amount of polyamine-conjugate reaching the DNA due to less competition by endogenous polyamines.

In summary, these *in vitro* studies demonstrated that conjugation of nitroimidazoles to polyamine carriers may help to target them to cells possessing the polyamine uptake system. In general 2-nitroimidazole conjugates had a higher affinity for the uptake system than 5-nitroimidazoles. In several cases, polyamine depletion markedly increased the toxicity of the conjugates but not the parent nitroimidazoles suggesting increased uptake of the compounds or increased DNA targeting had occurred. Compound 430 was a particularly potent inhibitor of spermidine uptake and although not itself toxic could potentially be used as a high affinity molecule carrier molecule to which cytotoxic drugs could be linked.

The structural requirements of compounds utilizing the uptake system were further investigated using a selection of spermidine and putrescine analogues. Their ability to inhibit spermidine uptake was found to be dependent on the type of polyamine backbone and the degree of substitution of the polyamine (table 3.2). In these studies, compounds with a spermidine backbone were found to be the best inhibitors of uptake and could withstand a high degree of substitution without losing their affinity for the uptake system. This was probably due to the fact that these compounds, despite substitution, are more likely to have two, sterically unhindered, amine groups available to interact with the polyamine receptor, a requirement for substrates of the uptake system (77,97,122,126). Spermidine was therefore chosen as the carrier molecule for further conjugates since it can be substituted and still leave two sterically unhindered nitrogen groups. It was chosen in preference to compound 430, which was a potent inhibitor of uptake but potentially more difficult to use in syntheses.

A conjugate of chlorambucil linked to the central nitrogen of spermidine by a spacer group (CLB-SPD) (fig 3.2) was tested for its uptake and toxicity to EATC. CLB-SPD was found to be a potent, competitive inhibitor of spermidine uptake in EATC (Ki =13.3μM). It also had a lower Ki than MGBG (Ki =34.7μM) suggesting that it was a better substrate than MGBG for the uptake system. The toxicity of CLB-SPD to EATC was increased by DFMO pretreatment which suggests that polyamine depletion enhanced the uptake of CLB-SPD into EATC. However it should be stressed that DFMO has both synergistic and antagonistic effects, which are not related to uptake, when used in combination with antineoplastic agents (160,161,162). DFMO induced polyamine depletion is reported to protect against chlorambucil toxicity in three out of four adenocarcinoma cell lines (286). In

this study, DFMO had a minor sensitizing effect on chlorambucil toxicity (fig 3.12) to EATC. Since the effect on the CLB-SPD conjugate toxicity was more marked it is likely that, in a similar manner to that seen for MGBG, enhanced uptake of CLB-SPD does play a role in its increased toxicity. A further point to consider is that polyamine depletion could reduce competition for DNA binding by endogenous polyamines, allowing more CLB-SPD to become associated with the DNA.

CLB-SPD was actually less toxic to control EATC than chlorambucil. This finding was initially disappointing since it was hoped that conjugation to a polyamine carrier would result in increased toxicity. However, in experiments with L1210 cells, a five fold enhancement in *in vitro* toxicity was seen for CLB-SPD compared to chlorambucil (fig 3.14) which was more encouraging. In addition, experiments using other cell lines (chapters 4 and 7) demonstrated that shorter incubation times (eg 1 hr) resulted in enhanced CLB-SPD toxicity compared to chlorambucil. This was probably due to a better utilization of the pharmacokinetic properties of the conjugate compared to chlorambucil over this shorter time period. Also, in some earlier experiments the effect of exposure time on chlorambucil toxicity to EATC was investigated (results not shown). An exposure time of 1 hr substantially reduced the toxicity of chlorambucil ($IC_{50}>50\mu M$). A 1 hr exposure to EATC may therefore have resulted in enhanced activity of the CLB-SPD conjugate compared to chlorambucil. In all experiments, a post exposure incubation period was required for DNA damage to be manifested as cell death.

In vivo, CLB-SPD demonstrated an acute CNS toxicity in mice at doses of 15mg/kg or greater (LD_{50} =17.5 mg/kg). The toxicity had a rapid onset with death occuring between 8 and 170 minutes. In contrast, CNS toxicity of chlorambucil, which was evident at concentrations 10-fold higher than CLB-SPD, was much milder and did not cause

death (results not shown). The CNS toxicity of CLB-SPD may be due to the presence of a polyamine uptake system in the nervous system (27) and it is interesting that spermidine and spermine are both reported to demonstrate acute lethal CNS toxicity in mice, although at much higher doses than seen for CLB-SPD (2). Polyamines (especially spermidine) may also be used in neurotransmission for which an uptake system would be important to remove released polyamines because their enzymic destruction is too slow. In addition, polyamines are also reported to be associated with the N-methyl-D-aspartate (NMDA) receptor in the CNS (287) and may enhance its activation or decrease its desensitization (2). High levels of spermidine are also reported to be associated with the myelin sheath of rat spinal nerves (27).

In initial *in vivo* antitumour studies against the EATC tumour model using low equimolar doses of chlorambucil and CLB-SPD, both compounds exhibited limited activity and an increase in survival time of about 10% was observed for both compounds (table 3.3). The activities of higher doses of the compounds were investigated, but due to the CNS toxicity of CLB-SPD multiple dosing was used in order to increase the total dose administered. Multiple dosing of CLB-SPD (2 x 12.5mg/kg) gave a small enhancement in lifespan compared to a single dose (5 mg/kg). In contrast, multiple chlorambucil dosing (2x5mg/kg) resulted in a large increase in survival time with four out of five animals surviving for at least 30 days (compared to 14 days for controls)(table 3.3). Overall, chlorambucil appeared to have greater *in vivo* activity than CLB-SPD against EATC (table 3.3) reflecting the results obtained *in vitro* with these compounds. Co-administration of spermidine with chlorambucil did not effect its activity suggesting that the spermidine moiety of CLB-SPD is not the cause of the inactivity of CLB-SPD. Some *in vivo* antitumour activity

was observed for compound 230 in the EATC tumour model but MGBG was inactive.

Neither CLB-SPD (10mg/kg) nor chlorambucil (20mg/kg) were active against the L1210 tumour *in vivo* despite the sensitivity of L1210 cells to both compounds *in vitro*. Treatment with MGBG (50mg/kg) and 5-fluoruracil (20 mg/kg), however, gave a greater than 40% increase in lifespan (table 3.4). An increase in lifespan of 25% or more is recognised as a positive antitumour effect in this tumour system (288). In agreement with this study, MGBG is reported in the literature as having *in vivo* activity against the L1210 tumour (182,289). In addition, 5-fluorouracil is the positive control compound used for the L1210 tumour by the National Institute of Health (NIH), USA.

In summary, a range of compounds were studied in for their *in vitro* toxicity and inhibition of spermidine uptake in EATC. A spermidine carrier was chosen as the most suitable for conjugation to cytotoxic agents. One lead compound, a CLB-SPD conjugate was tested for its *in vitro* and *in vivo* activity in EATC. It was a good inhibitor of spermidine uptake and data suggests it utilizes the polyamine uptake system. The toxicity of CLB-SPD to EATC *in vitro* was poorer than that of chlorambucil but this may have been due to poor choice of exposure time. Polyamine depletion increased the toxicity of the conjugate. ^{3}H-Thymidine incorporation gave similar results to those obtained in clonogenic assays for a number of compounds and would therefore seem a suitable method for measuring *in vitro* toxicity. *In vivo* CLB-SPD was not particularly active against EATC or L1210 tumour models, although chlorambucil was active against EATC. In spite of the disappointing results obtained with CLB-SPD in EATC it was decided to study the effects of the conjugate on a variety of other cell lines since it showed promising activity against L1210 cells. These results are presented in the following chapters.

CHAPTER 4 - Comparison of the *in vitro* and *in vivo* activity of CLB-SPD conjugate and chlorambucil in ADJ/PC6 plasmacytoma

4.1 Introduction

Chlorambucil is one of the best tolerated p.o. alkylating agents and is widely used in the treatment of chronic lymphocytic leukemia, lymphomas and ovarian carcinoma (200). It enters tumour and other cells, including chronic lymphocytic leukemic lymphocytes, by diffusion (211). The common dose limiting toxicity of chlorambucil is irreversible bone marrow damage which leads to hematopoietic suppression (200). The main reason for this toxicity is its lack of selectivity for tumour tissue. Attempts have been made to increase tumour selectivity of alkylating agents by conjugating them to tumour specific antibodies. However, in the majority of cases, coupling of alkylating agents to antibodies results in reduced alkylating activity, although tumour specificity is increased (240).

It has been suggested that N^4-spermidine derivatives may also be able to act as vector molecules for the selective delivery of small anticancer agents (97,121), since spermidine can be modified extensively at the N^4 position and still be accumulated by the polyamine uptake system (97). A chlorambucil-spermidine conjugate was therefore synthesized by conjugating chlorambucil to the central nitrogen of spermidine. A spacer group was used between the two compounds to reduce the steric hinderance of the central N^4 atom (fig 3.2).

In chapter 3, the *in vitro* and *in vivo* activities of CLB-SPD against EATC were described. It was proposed that CLB-SPD would be more toxic than chlorambucil to these cells, which possess a well documented uptake system, as the result of increased uptake. The toxicity of the conjugate to EATC was, however, actually poorer

than chlorambucil.

Since EATC did not seem particularly sensitive to chlorambucil, the murine ADJ/PC6 plasmacytoma, which is sensitive to alkylating agents (290), was chosen for use in further studies. It was hoped that greater success would be achieved using a more sensitive cell line. The ADJ/PC6 plasmacytoma, which was originally derived from a solid B-cell tumour (270), grows *in vitro* and *in vivo* allowing comparitive studies to be performed. The L1210 cell line, which was sensitive to CLB-SPD *in vitro* (chapter 3) was not used since it proved difficult to grow in culture.

Following confirmation that a polyamine uptake system was present in the ADJ/PC6 cells, the affinity of CLB-SPD for the uptake system was investigated. The *in vitro* and *in vivo* toxicities of CLB-SPD and chlorambucil to this tumour were also compared. CLB-SPD was shown to have a high affinity for the polyamine uptake system and demonstrated greater *in vitro* toxicity than chlorambucil. However, the encouraging *in vitro* activity was not fully realized *in vivo*.

4.2 Results

4.2.1 Characterisation of spermidine uptake in ADJ/PC6 cells; the effects of temperature and polyamine depletion.

Prior to the use of ADJ/PC6 plasmacytoma cells in further studies with CLB-SPD, the kinetics of ^{14}C-spermidine uptake in these cells was investigated to confirm that a polyamine uptake system was present. The ADJ/PC6 cells were found to have a temperature and concentration dependent uptake system for spermidine (fig 4.1). At 37°C, kinetic parameters for spermidine uptake were Km = 0.23 ± 0.05 μM (n=3) and Vmax = 2.84 ± 0.13 pmoles/10^5cells/min.

Polyamine depletion, achieved by preincubation of the cells with DFMO (0.5mM, 72 hr) resulted in an increase in the rate of spermidine uptake at 37°C (fig 4.2). Under these conditions a Km of 0.2μM and Vmax of 4.3 pmoles/10^5cells/min were obtained. Kinetic parameters were calculated as described in 3.2.1.

4.2.2 The effect of MGBG on spermidine uptake in ADJ/PC6 cells and its *in vitro* toxicity to control and polyamine depleted cells.

MGBG (10μM) inhibited competitively the uptake of ^{14}C-spermidine into ADJ/PC6 cells at 37°C (fig 4.3). A Ki value of 3.4 μM was calculated from Lineweaver-Burk plots.

The *in vitro* toxicity of MGBG (24 hr exposure) to control and polyamine depleted ADJ/PC6 cells was also investigated. DFMO pretreatment (0.5mM, 72 hr) resulted in an approximate 3-fold increase in toxicity of MGBG (fig 4.4)(IC$_{50}$ (control) = 2.5μM, IC$_{50}$ (DFMO) = 0.8μM)

Fig 4.1 Kinetics of spermidine uptake in ADJ/PC6 cells and the effect of temperature.

A

B
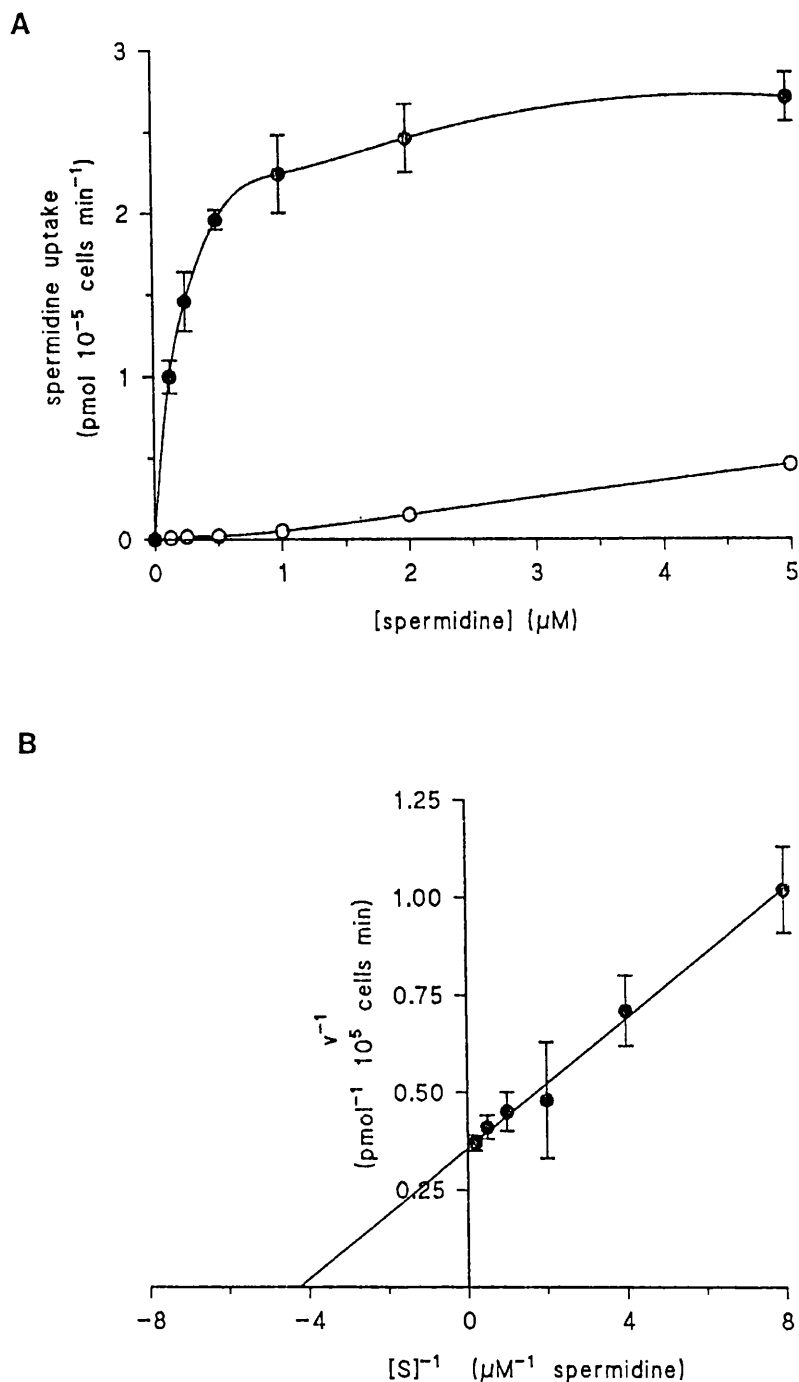
ADJ/PC6 plasmacytoma cells (1×10^5) were incubated for 30 min with ^{14}C-spermidine (0.125 - 5.0 μM) at 37°C (●) and 4°(o). The rate of spermidine uptake is presented as both linear (a) and Lineweaver-Burk (b) plots. Data represent the mean ± s.e.m. (n=3) for 37°C and the mean of 2 experiments at 4°C. All experiments were performed in duplicate.

Fig 4.2   The effect of DFMO on the kinetics of spermidine uptake in ADJ/PC6 cells.

A

B
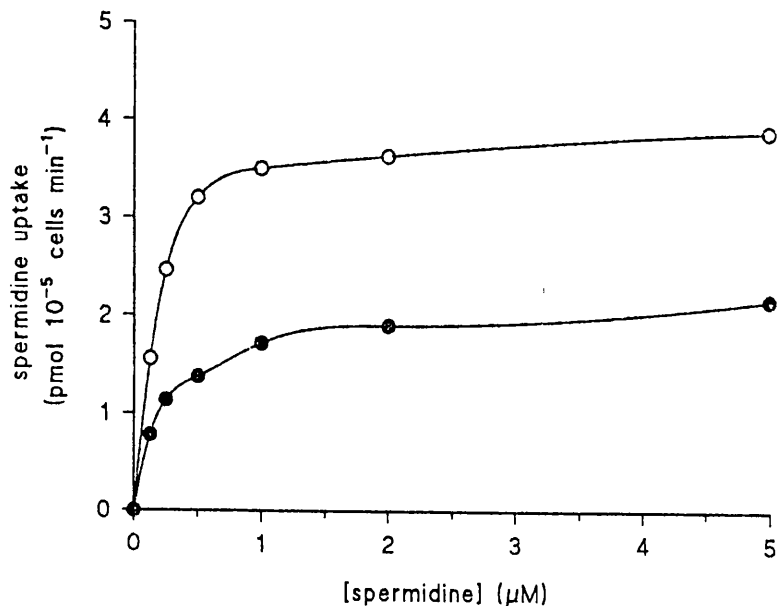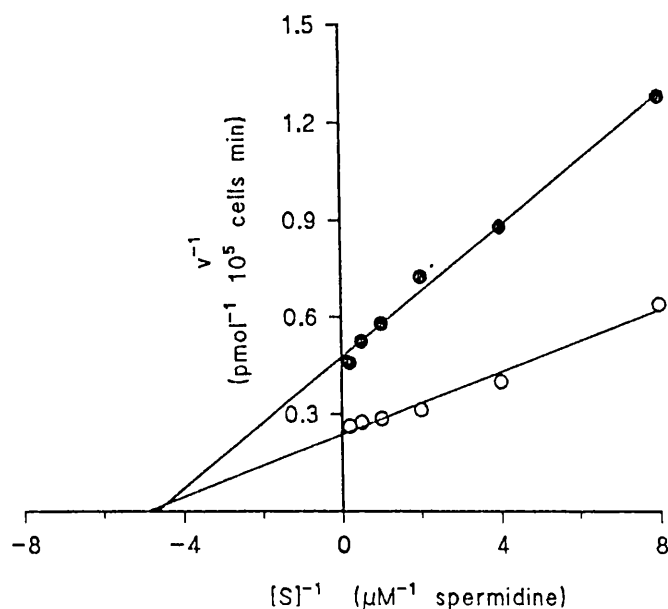
ADJ/PC6 cells (1×10^5/ml) were preincubated in the presence or absence of DFMO (0.5mM) for 72 hr.   Cells were diluted and 1×10^5 control (●) or DFMO pretreated (○) cells were incubated with ^{14}C-spermidine (0.125 - 5.0 μM) for 30 min at 37°C.   Results are presented as both linear (a) and Lineweaver-Burk (b) plots.   Km and Vmax values were calculated.   The data shown are representative of two experiments performed in duplicate.

Fig 4.3 The effect of MGBG on the kinetics of spermidine uptake.
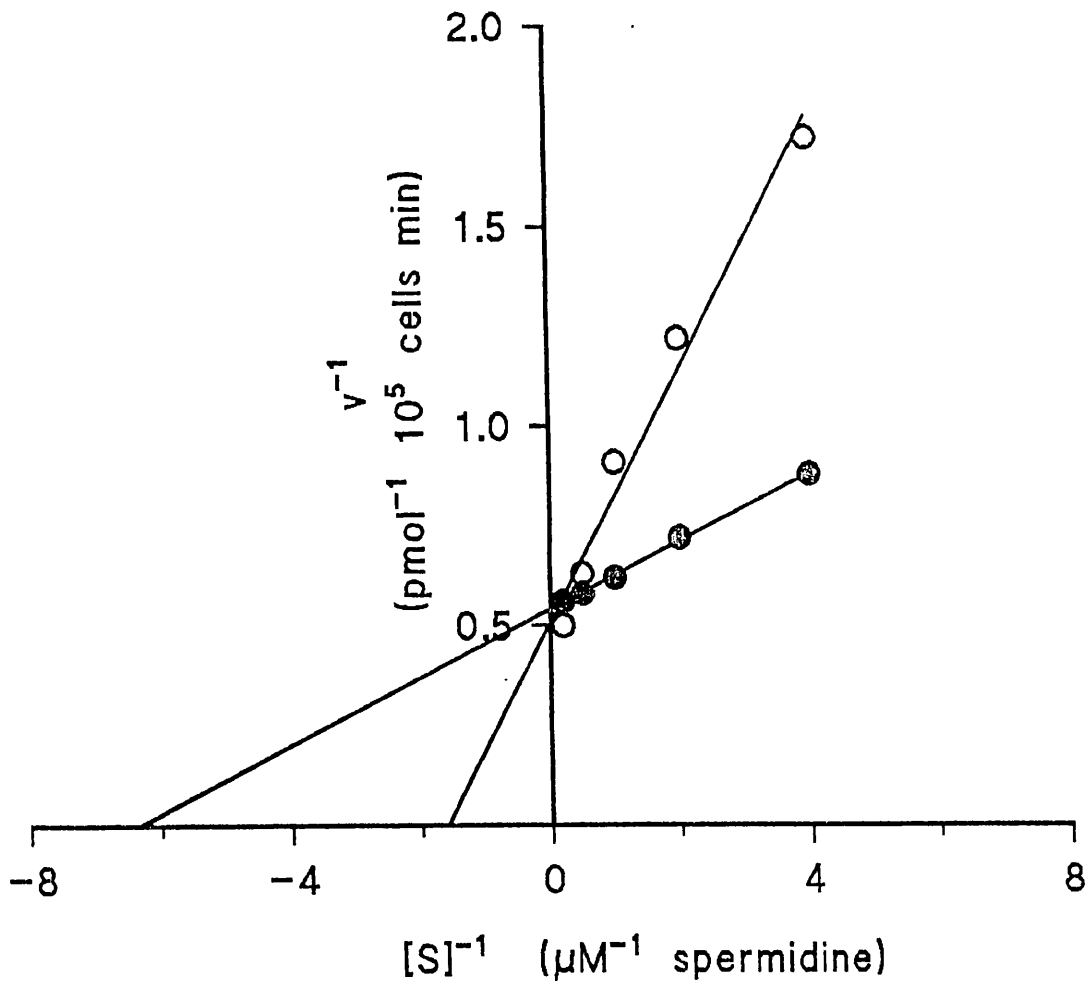
The uptake of ^{14}C-spermidine (0.25 - 5.0μM) in ADJ/PC6 cells (1x10^{5}) was measured at 37°C either alone (●) or in the presence of MGBG (10μM)(o). Results are presented as a Lineweaver-Burk plot from which Ki values were calculated. Data represent the means of 2 experiments, each performed in duplicate.

Fig 4.4 The effect of DFMO on the *in vitro* toxicity of MGBG to ADJ/PC6 cells
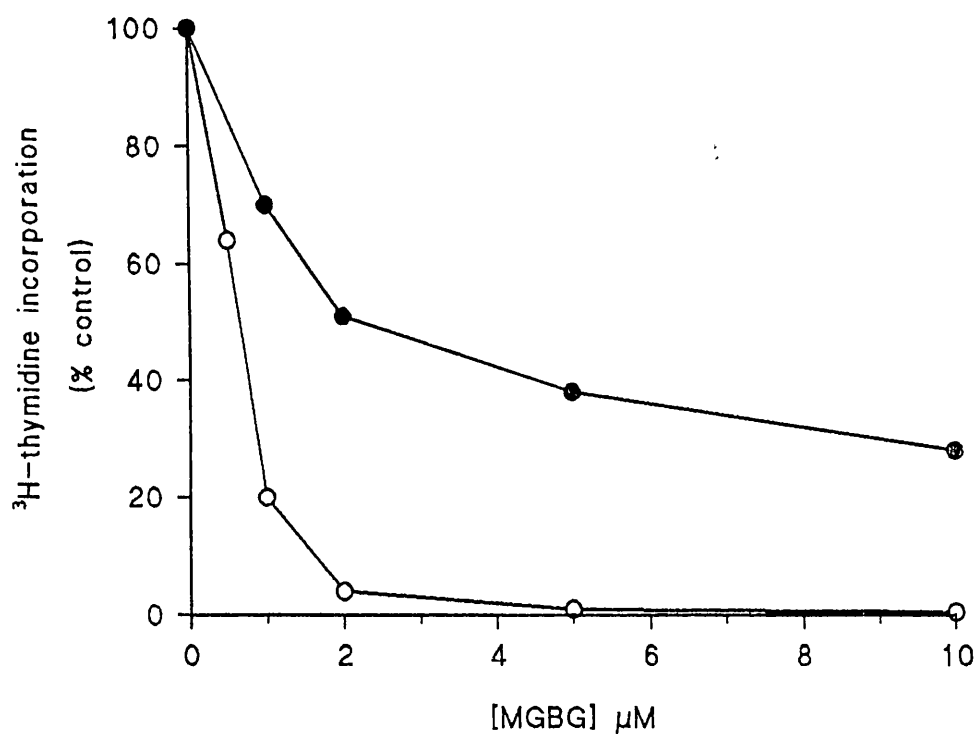
Control (●) and DFMO pretreated (O) ADJ/PC6 cells (1-2x10^{5}/ml) were incubated with MGBG (0-10μM) for 24hr and fresh medium added for 48hr. Cell viability was assessed by measuring ^{3}H-thymidine incorporation into DNA. Data represent the means of two experiments performed in triplicate.

4.2.3 Investigation of the effects of chlorambucil-spermidine and chlorambucil on spermidine uptake in ADJ/PC6 cells.

CLB-SPD (2 and 10 μM) inhibited competitively the uptake of ^{14}C-spermidine into ADJ/PC6 cells (fig 4.5a). A Ki value of [*]0.80 ± 0.07μM (n=3) was obtained for CLB-SPD. Chlorambucil had no effect on spermidine uptake (fig 4.5b).

4.2.4 Comparison of the *in vitro* toxicity of chlorambucil-spermidine and chlorambucil to ADJ/PC6 cells, the effect of polyamine depletion and exogenous spermidine.

The toxicities of CLB-SPD and chlorambucil to control and DFMO pretreated (0.5mM, 72 hr) ADJ/PC6 cells were assessed by their abilities to inhibit ^{3}H-thymidine incorporation. CLB-SPD was approximately 30-fold more toxic than chlorambucil after a 1 hr exposure to control cells as indicated by the IC_{50} values (table 4.1).When exposed for 72 hr, CLB-SPD was only 7-fold more toxic than chlorambucil (table 4.1).

Clonogenic assays confirmed the enhanced toxicity of CLB-SPD compared to chlorambucil in control cells (fig 4.6) where a 50-fold difference in toxicity was observed following a 1 hr exposure (IC_{50}(CLB-SPD) = 0.075μM, IC_{50}(chlorambucil)= 3.8μM) and a 4-fold difference (IC_{50} (CLB-SPD) = 0.04μM, IC_{50}(chlorambucil) = 0.15μM) following a 24hr exposure (fig 4.6).

DFMO itself gave an approximate 50% decrease in ^{3}H-thymidine incorporation compared to control cells and this was taken into account when calculating IC_{50} values. Polyamine depletion with DFMO increased the toxicity of CLB-SPD for both exposure times (table 4.1). This resulted in a striking 170-fold increase in toxicity of CLB-SPD compared to chlorambucil following a 1 hr exposure (table 4.1).

[*]mean ± s.e.m.

**Fig 4.5 The effects of CLB-SPD (a) and chlorambucil (b)
on the kinetics of spermidine uptake.**

A
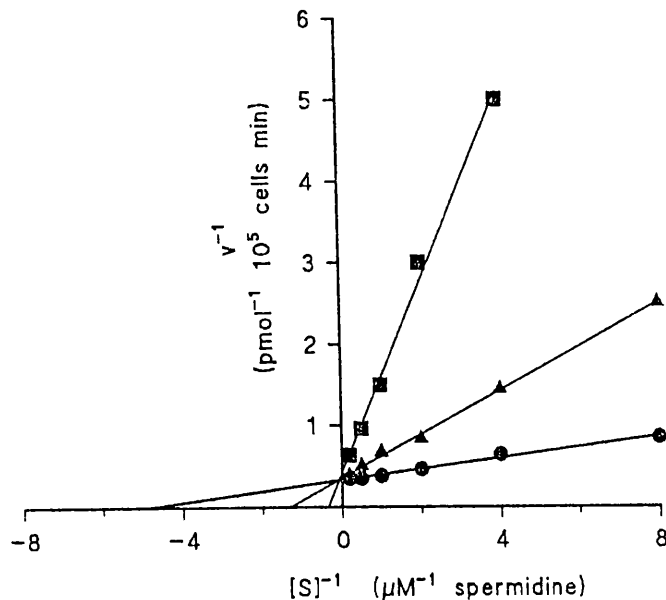
B
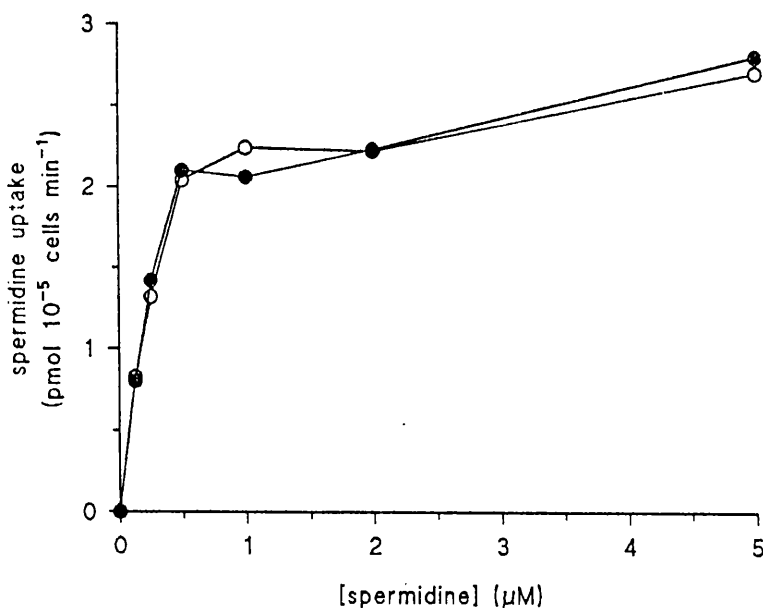
The uptake of ^{14}C-spermidine (0.125 - 5.0 μM) in ADJ/PC6
cells (1x10^5) was measured at 37°C either alone(●) or in
the presence of CLB-SPD (2μM (▲) or 10μM (■)) (a) or
chlorambucil (20μM (O)) (b). Results for CLB-SPD are
presented as a Lineweaver-Burk plot and data shown is
representative of three experiments performed in
duplicate. For chlorambucil, results are presented as a
linear rate plot.

Table 4.1 *In vitro* toxicity of chlorambucil and chlorambucil-spermidine conjugate to ADJ/PC6 cells following 1 or 72 hr exposure.

| Compound | IC_{50} μM | | | |
| | 1 h exposure | | 72 h exposure | |
	Control	DFMO	Control	DFMO
chlorambucil	8.9 ± 1.19	22.2 [b]	0.22 ± 0.02	0.31 ± 0.02
chlorambucil-spermidine	0.29 ± 0.04	0.13 ± 0.03	0.03 ± 0.003	0.01 [b]
fold-difference	30	170	7	31

Cells were exposed to the test compounds for either 72 hr or 1 hr followed by the addition of fresh medium for 71 hr. ^{3}H-thymidine was added for the last 2 hr of the incubation.

[a]IC_{50}, concentration of compound giving a 50% reduction in ^{3}H-thymidine incorporation. Values are mean ± SEM of at least 3 observations except as noted.
[b] n=2

Fig 4.6 *In vitro* toxicity of CLB-SPD and chlorambucil as assessed by clonogenic assays
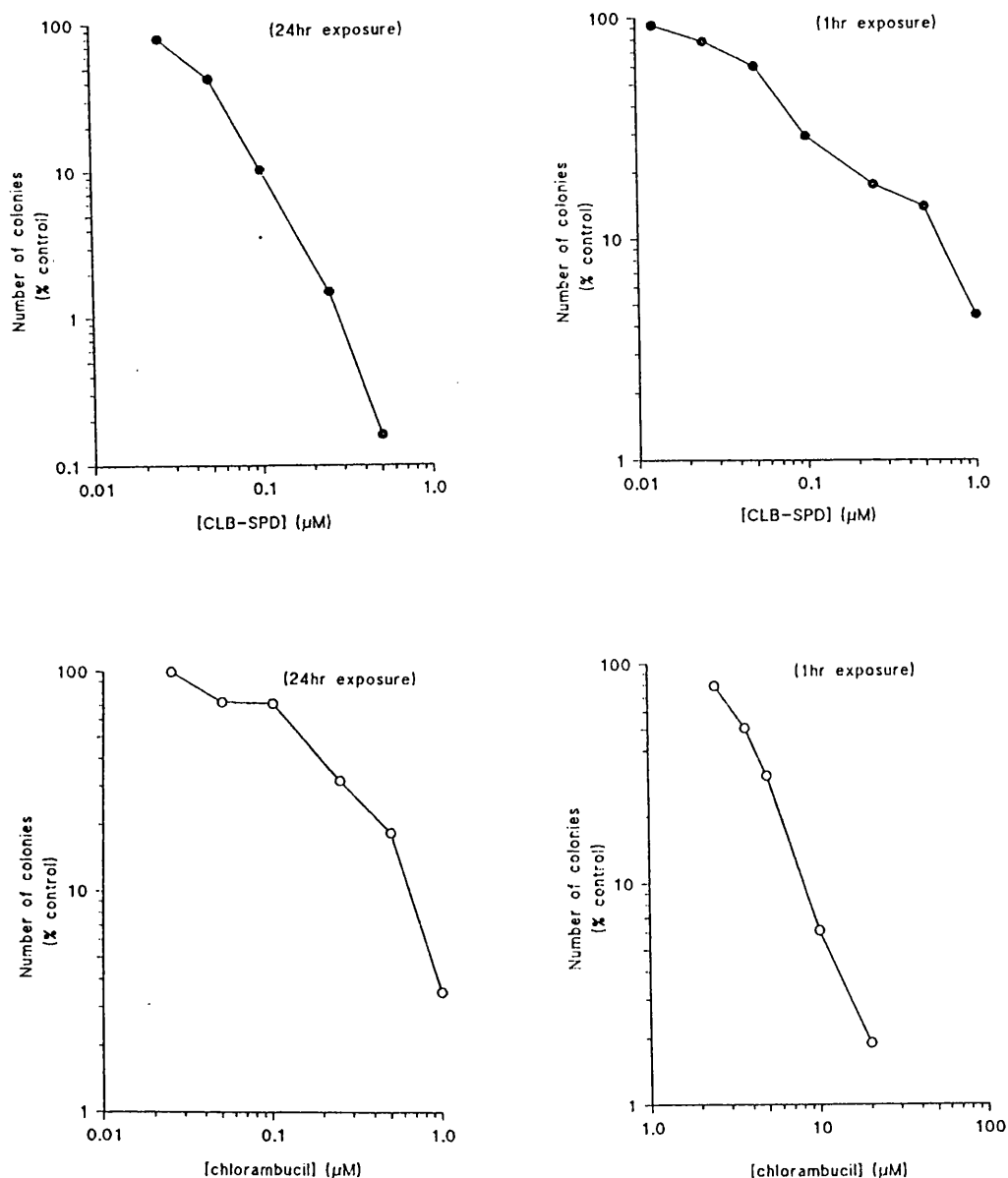
ADJ/PC6 cells (2×10^5/ml) were incubated with CLB-SPD(●) (0 - 1.0 μM) or chlorambucil(○) (0 - 20 μM) for 1 or 24hr. Cells were resuspended at various densities in a fresh medium/agarose mixture and cultured for a further 2-3 weeks. The number of colonies present after this time period were counted and expressed as a percentage of control (untreated) cells. Data are the means of two experiments performed in duplicate using 3 seeding densities.

Addition of spermidine (1, 10 and 20 μM) to the incubation medium of the 1 hr exposure experiment reduced the toxicity of CLB-SPD (fig 4.7a) but had no effect on the toxicity of chlorambucil (fig 4.7b), as measured by ^{3}H-thymidine incorporation.

4.2.5 *In vivo* antitumour activity of chlorambucil-spermidine and chlorambucil against the ADJ/PC6 plasmacytoma.

A single i.p. administration of both chlorambucil and CLB-SPD to mice bearing the ADJ/PC6 plasmacytoma caused a marked dose dependent reduction in tumour weight. The *in vivo* antitumour activity of CLB-SPD was approximately 2-fold greater than chlorambucil when expressed on a molar basis, as indicated by the doses inhibiting tumour growth by 90% (ED_{90}) (table 4.2). Due to the lower LD_{50} of CLB-SPD (discussed in 3.3), the therapeutic index of the conjugate was actually lower than chlorambucil (table 4.2). It should also be noted that in earlier experiments using a batch of CLB-SPD which contained dichlorambucil-spermidine (ie 2 chlorambucil molecules conjugated to spermidine) as an impurity (approximately 15%), greater activity was observed. The mass spectra of pure CLB-SPD and of a less pure sample containing dichlorambucil-spermidine conjugate are shown in fig 4.8.

In a preliminary experiment, the effect of administering CLB-SPD by continuous infusion for 12 hr via a tail vein cannula was investigated. This was carried out in an attempt to utilize the pharmacokinetics of the polyamine uptake system to enhance delivery of the CLB-SPD conjugate and increase its antitumour activity. The dose response curve did appear to be moved slightly to the left (fig 4.9), suggesting a possible increase in antitumour activity, although statistical analysis (student's t-test) showed it to be non-significant.

Fig 4.7 The effect of exogenous spermidine on the toxicity of CLB-SPD (a) and chlorambucil (b).

A

B
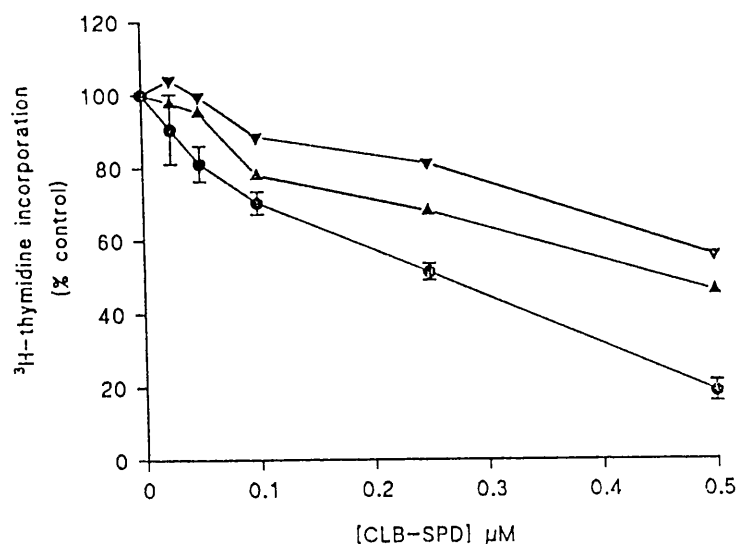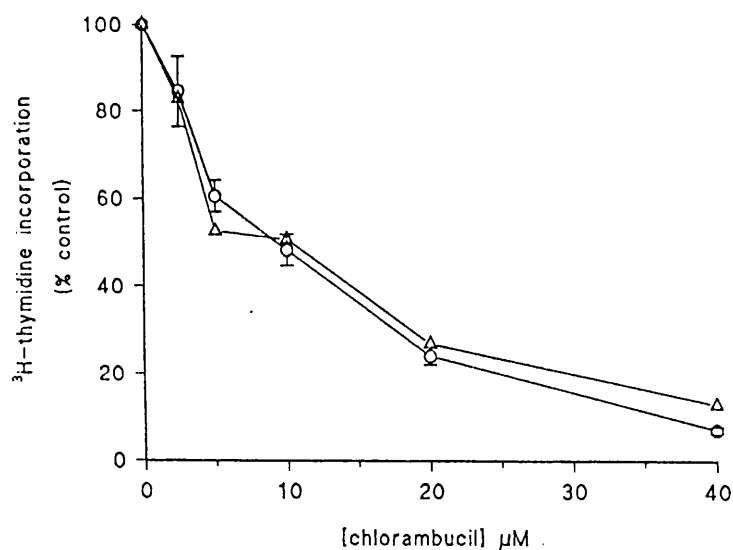
ADJ/PC6 cells (1×10^5/ml) were incubated for 1 hr with CLB-SPD (●) (0 - 0.5 μM) or chlorambucil (○)(0 - 40 μM) alone or in the presence of spermidine (1μM (▲), 10μM (▼) and 20μM (△)). Fresh medium was added to the cells and cell viability was assessed by measuring ^{3}H-thymidine incorporation into DNA during the final 2 hr of the incubation. Data shown are representative of two experiments performed in duplicate except for CLB-SPD and chlorambucil alone where data represent the mean ± s.e.m. (n=5).

Table 4.2 *In vivo* antitumour activity of chlorambucil and chlorambucil-spermidine conjugate to ADJ/PC6 plasmacytoma.

Compound	ED_{90}[a] μmoles/kg	LD_{50}[a] μmoles/kg	Therapeutic index
chlorambucil	9.6 ± 1.4	125 [b]	13.0
chlorambucil-spermidine	4.6 ± 0.7	27.6 [c]	6.0
fold-difference	2.1	4.5	2.1

Mice innoculated with ADJ/PC6 plasmacytoma were treated with a single i.p. injection of chlorambucil or chlorambucil-spermidine. At the end of the study tumour weights of control and treated animals were compared.

[a]ED_{90};dose of compound giving a 90% reduction in tumour size; LD_{50},50% lethal dose.
[b]Chronic toxicity.
[c]Acute toxicity.

Fig. 4.8 Mass specta of pure CLB-SPD (a) and impure CLB-SPD containing dichlorambucil-spermidine conjugate (b).

A
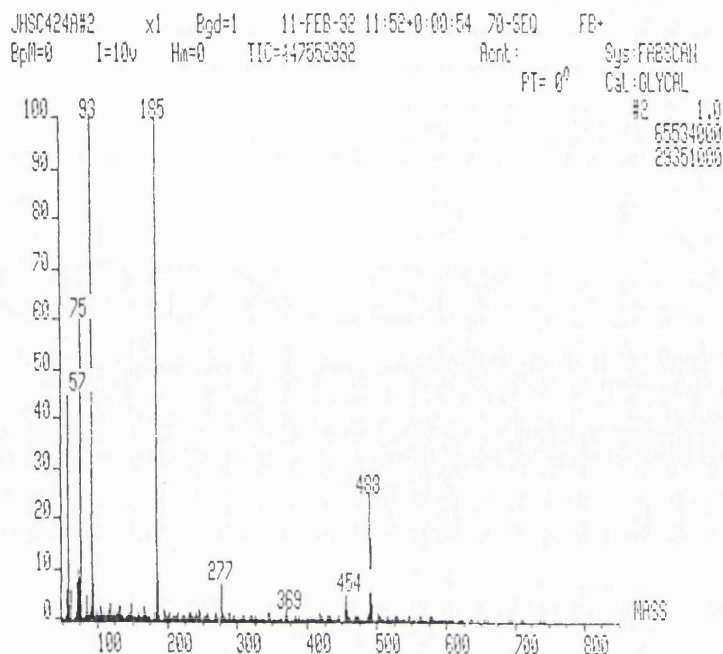
B
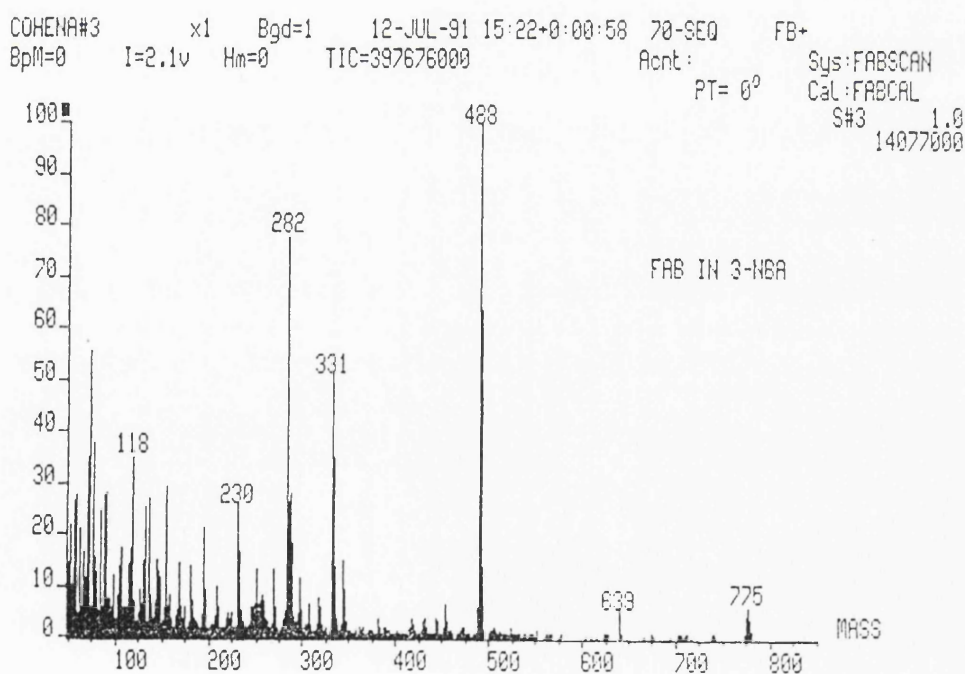
M^+ (CLB-SPD) = 488

M^+ (diCLB-SPD) = 775

Fig 4.9   Effect of continuous infusion compared to i.p administration on the *in vivo* antitumour activity of CLB-SPD conjugate to ADJ/PC6 plasmacytoma.
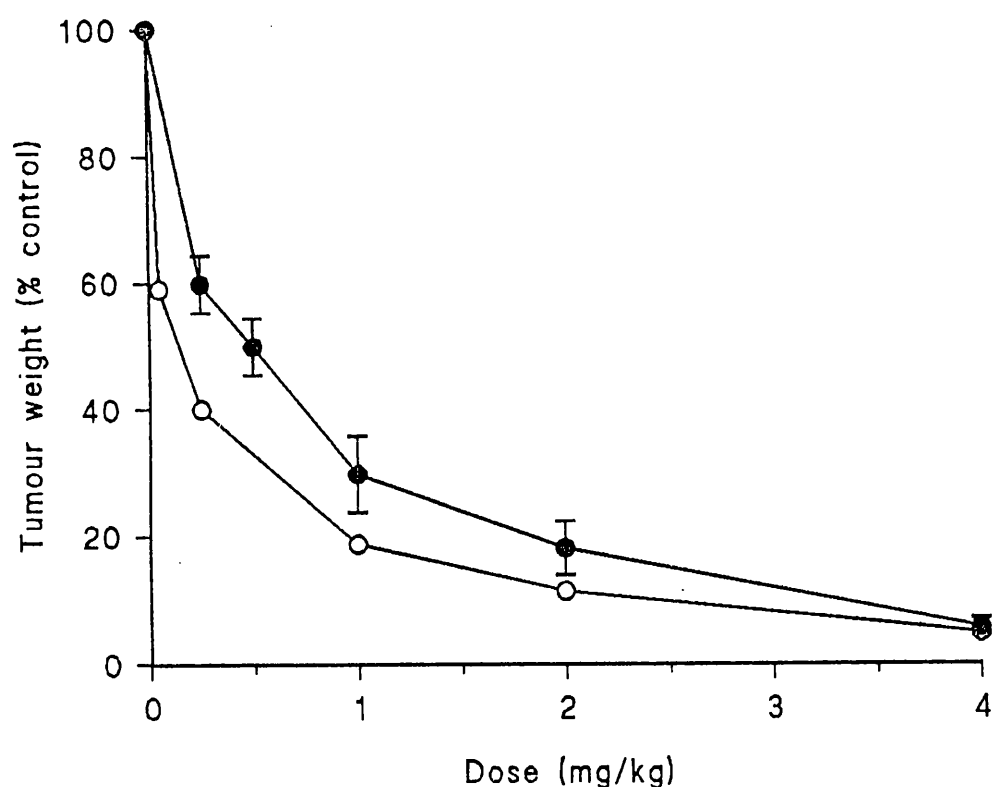
Mice innoculated with ADJ/PC6 plasmacytoma were treated on day 20 of the study with CLB-SPD either by a single i.p. injection (●) or by continuous i.v. infusion (O) into the tail vein.

Results for i.p. administration represent mean ± s.e.m. (n=5) and for the infusion experiment, one experiment only (4 mice per dose).

4.4 Discussion

In this study, the ADJ/PC6 plasmacytoma was shown to possess a saturable, temperature dependent uptake system for spermidine (fig 4.1). Polyamine depletion with DFMO resulted in a 2-fold increase in the Vmax for spermidine uptake, but no change in Km. This was similar to the effect seen in polyamine depleted EATC (chapter 3), although in these latter cells, a much greater (6-fold) increase in Vmax was observed following polyamine depletion. MGBG also inhibited competitively spermidine uptake in the ADJ/PC6 cells (fig 4.3). These results demonstrated that ADJ/PC6 cells have a polyamine uptake system similar in its kinetic parameters to those reported for other tumour cells (56) and EATC (chapter 3). It was therefore considered a suitable model for testing the CLB-SPD conjugate.

CLB-SPD inhibited competitively the uptake of spermidine into ADJ/PC6 cells (fig 4.5). The affinity of CLB-SPD for the uptake system of the ADJ/PC6 cells was greater than that of MGBG (Ki=0.8μM and 3.4 μM respectively). Similar observations have been reported for a number of N^4-spermidine derivatives (121) and confirm previous indications that N^4-derivatives of spermidine are likely to retain the uptake characteristics of spermidine (121). Chlorambucil did not inhibit spermidine uptake (fig 4.5) which indicates that it did not have an affinity for the polyamine uptake system.

The studies were extended to compare the *in vitro* toxicity of CLB-SPD and chlorambucil to the ADJ/PC6 cells. The results of these studies demonstrated how, under a variety of exposure conditions, conjugation of chlorambucil to spermidine resulted in an increase in *in vitro* toxicity, compared to non-conjugated parent compound (table 4.1). Clonogenic assays confirmed these results (fig 4.6).

The enhanced *in vitro toxicity* of CLB-SPD compared to chlorambucil may have been due to a number of reasons, including increased uptake of the conjugate into the cells via the polyamine uptake system which would result in higher intracellular concentrations than could be achieved with chlorambucil alone. The observation that CLB-SPD was a competitive inhibitor of spermidine uptake (fig 4.5), with a low Ki, indicates that it has a high affinity for the polyamine uptake system. This, together with the observation that exogenous spermidine reduced the toxicity of CLB-SPD (but not chlorambucil) (fig 4.7) in a concentration dependent manner, possibly by inhibiting uptake of the conjugate, supports the hypothesis that CLB-SPD utilizes the polyamine uptake system. In a similar manner, the ability of spermidine to inhibit the uptake and subsequent toxicity of MGBG, which utilizes the same uptake system, was seen in EATC in chapter 3 and is also reported in the literature (154).

When exposed to the ADJ/PC6 cells for 1 hr, CLB-SPD was 30-fold more toxic than chlorambucil, but only 7-fold more toxic after a 72 hr exposure (table 4.1). This effect was also seen using clonogenic assays to measure toxicity (fig 4.6). The difference in toxicity for the two exposure times was mainly the result of a large reduction in the toxicity of the chlorambucil following a 1 hr exposure compared to a 72 hr exposure. The shorter exposure time, which may reflect more closely the exposure time *in vivo* (255), should facilitate the uptake of the CLB-SPD conjugate by the polyamine uptake system compared to chlorambucil. In addition, CLB-SPD may remain in an intracellular pool, even after removal of drug from the medium, due to the nature of the polyamine carrier which has a high affinity for cellular macromolecules such as RNA (3) and specific polyamine binding sites (120,180). As a result of this, as cells go through to a more sensitive part of the cell cycle,

the CLB-SPD would still be available to interact with DNA, resulting in enhanced toxicity. In contrast, following the 1 hr exposure, chlorambucil would simply diffuse out of the cell and fewer cells in the sensitive part of the cell cycle would come into contact with the alkylating agent than would occur for the longer exposure time.

Especially striking was the 170-fold increase in toxicity of CLB-SPD compared to chlorambucil following a 1 hr exposure to polyamine depleted cells (table 4.1). This was due to a combination of both increased toxicity of CLB-SPD and a decreased toxicity of chlorambucil. DFMO also reduces the toxicity of chlorambucil in a number of human adenocarcinoma cell lines (286). The observations with chlorambucil were therefore in keeping with these results.

An interesting observation was the relationship between the degree of polyamine depletion, as assessed by the increase in spermidine uptake, and the enhancement in toxicity of MGBG and CLB-SPD. In ADJ/PC6 cells polyamine depletion resulted in a 2-fold increase in the Vmax of spermidine uptake and also a 2-fold increase in the total amount of spermidine uptake over a 1 hr time period (results not shown). The toxicities of CLB-SPD and MGBG were also increased 2-3 fold by polyamine depletion, which suggests a 2-3 fold increase in uptake of these compounds. By comparison, in EATC, polyamine depletion resulted in a 6-fold increase in the Vmax for spermidine uptake and the toxicity of MGBG and CLB-SPD were increased 10 and 5 fold respectively (chapter 3). These results are consistent with the hypothesis that the CLB-SPD conjugate utilizes the polyamine uptake system in a similar manner to MGBG.

Experiments carried out in collaboration with Dr J. Hartley (Dept. Oncology, University College and Middlesex Hospital School of Medicine, London) demonstrated that the CLB-SPD conjugate was approximately

10,000 fold more effective at forming crosslinks with naked DNA than chlorambucil (results not shown) (291). This was a property unique to the conjugate inasmuch as equimolar mixtures of chlorambucil and spermidine did not give enhanced alkylation over chlorambucil alone. In fact, inhibition of alkylation was observed (291). DNA affinity binders, such as polyamines, have been shown previously to dose dependently inhibit the nitrogen mustard alkylation of guanine N-7 positions in the major groove, the primary site of base alkylation by such agents (292).

Studies have shown that the polycationic nature of spermidine gives it a high affinity for DNA (259,5). In addition it has recently been shown that while constrained to remain close to DNA, the polyammonium cations retain a high degree of freedom of motion within the polycation-DNA complex (293). Clearly the conjugation of chlorambucil to spermidine has coupled high affinity with the ability of the bifunctional alkylating group to locate sites suitable for efficient DNA interstrand cross-linking in naked DNA. If this enhanced DNA affinity of the CLB-SPD conjugate occurs in cells, its enhanced *in vitro* toxicity, compared to chlorambucil, may be due to the high affinity of the polyamine moiety for DNA rather than uptake differences. Following polyamine depletion, one would envisage CLB-SPD having even greater affinity for DNA due to less competition for binding by endogenous polyamines and cations. This could explain why polyamine depletion increased the toxicity of the conjugate.

It should also be considered that as initial DNA binding will be dictated by the spermidine moiety, the major site of interstrand cross-linking may not necessarily now be between guanine N-7 sites in the major groove as characteristic of nitrogen mustards. The precise location of the alkylation reactions and the relative importance of these lesions to the cytotoxicity

141

of the conjugate remain to be determined. It has also
been suggested that in addition to forming DNA
interstrand cross-links, alkylating agents may also
alkylate and cause extensive alterations in plasma
membranes (217,218) and may also uncouple DNA and histone
synthesis (218). It is feasible that one of these
alternative mechanisms could occur for CLB-SPD.

Despite the promising *in vitro* results, *in vivo*
antitumour studies showed that the conjugate was only 2-
fold more potent than chlorambucil against the ADJ/PC6
plasmacytoma (table 4.2). In addition, the therapeutic
index of the conjugate was actually lower than
chlorambucil due to the acute CNS toxicity of CLB-SPD
(which is discussed in chapter 3). The minor increase in
in vivo activity did not reflect the 30-fold increase
observed *in vitro* with control ADJ/PC6 cells or the
10,000 fold increased reactivity with naked DNA. The
reasons for this were unclear and may include several
possibilities such as alterations in metabolism,
pharmacokinetics, intracellular distribution, DNA binding
and repair. It was also an interesting observation that
greater *in vivo* activity was seen with a batch of CLB-SPD
which contained a small amount of dichlorambucil-
spermidine (15%) as an impurity (fig 4.8). The
significance of this is discussed in chapter 8.

The critical importance of pharmacokinetics in
maximizing the therapeutic benefits from active uptake
systems has been previously discussed (267). Provided
that the tumour cells have a lower Km (ie higher
affinity) for the uptake of the cytotoxic conjugate than
normal cells *in vivo*, slow infusion of the conjugate at
concentrations near its Km may lead to selective
accumulation in the tumour cells. This should in turn
result in greater antitumour activity plus a reduction in
the acute toxicity observed with a bolus dose, leading to
an increase in the therapeutic index. In a preliminary
study in which mice bearing the ADJ/PC6 plasmacytoma were

infused with CLB-SPD over a 12 hr period, the dose
response for tumour kill was shifted to the left compared
to i.p. dosing. However, this shift was marginal and
statistically non-significant (fig 4.9) and suggests that
kinetics do not play as an important role in determining
the activity of the conjugate as originally proposed.

In conclusion, the CLB-SPD conjugate showed
greater antitumour activity both *in vitro* and *in vivo*
than chlorambucil. This was probably due to increased
uptake, increased affinity for DNA or a combination of
the two. A remarkable 10,000 fold increased
effectiveness for cross-linking DNA was observed
following conjugation of chlorambucil with spermidine.
This increased activity was not fully realized *in vivo*
and further studies comparing tissue delivery and
metabolism of the compounds were required. This problem
is addressed in the following chapter using a novel HPLC
system developed for this aim.

CHAPTER 5 - Comparison of the *in vivo* metabolism, tumour uptake and tissue distribution of CLB-SPD and chlorambucil.

5.1 Introduction

In chapter 4, the *in vitro* and *in vivo* activity of a CLB-SPD conjugate was compared to chlorambucil in the ADJ/PC6 plasmacytoma. CLB-SPD had a high affinity for the polyamine uptake system and was 30-fold more toxic than chlorambucil *in vitro*. It was also 10,000-fold more reactive with naked DNA than chlorambucil. *In vivo*, however, the CLB-SPD conjugate was only 2 fold more active than chlorambucil.

Reasons for the reduced activity include the possibility that the conjugate may be metabolised *in vivo*, perhaps by cleavage of the amide linkage, releasing free chlorambucil. In addition, delivery of CLB-SPD to the tumour tissue *in vivo* may not be enhanced, compared to chlorambucil, as hypothesized. These possibilies could be addressed by measuring plasma and tissues levels of CLB-SPD and chlorambucil following their administration to mice. To achieve this, an HPLC method for the determination of CLB-SPD and chlorambucil in biological samples was required.

Several attempts to measure CLB-SPD and chlorambucil were made using modifications of existing HPLC methods for chlorambucil (211) and polyamine (294) analysis. The gradient elution method used by Bank *et al.* for measuring chlorambucil (211) was unsuitable since severe peak tailing was observed with CLB-SPD and peak areas were erratic and unrelated to the amount of material injected. The number of injections made previously on the column also affected the peak area obtained. The suitability of a method used by Hunter and Fairlamb for polyamine analysis (294) was also investigated. Polyamine quantitation normally involves

their pre- or post- column derivatisation to allow their
detection (156,293,295). This was unnecessary since both
CLB-SPD and chlorambucil could be detected by their UV
absorbance at 260nm. An adaptation of the method used by
Hunter and Fairlamb (294) gave good separation of
chlorambucil and CLB-SPD but the peaks were fairly broad,
reducing the sensitivity of the method. In addition the
presence of camphorsulphonic acid in the eluent posed a
hazard due to its irritant properties. Therefore a more
sensitive and less hazardous HPLC system for CLB-SPD and
chlorambucil analysis was developed.

This new HPLC method was used to investigate the
plasma concentrations, half lives and metabolic profiles
of CLB-SPD and chlorambucil following their i.p.
injection in mice and to determine whether the amide
linkage of the conjugate was stable. The method was also
used to compare the *in vitro* and *in vivo* uptake of CLB-
SPD and chlorambucil in the ADJ/PC6 plasmacytoma. This
was to determine whether increased uptake of CLB-SPD,
compared to the non-conjugated parent compound
chlorambucil, occurred. The kinetics of CLB-SPD uptake
in vitro were also determined. In addition, the *in vivo*
tissue distribution of CLB-SPD and chlorambucil were
compared to determine whether any enhanced accumulation
of CLB-SPD compared to chlorambucil was tumour specific.

5.2 Methods

5.2.1 High-Performance Liquid Chromatography conditions

HPLC was carried out using a Varian 5000 liquid chromatograph with UV detection at 260nm. CLB-SPD, chlorambucil and some chlorambucil metabolites were separated using a 250 x 5 mm i.d Hypersil ODS (5μm) column (Shandon Scientific, Runcorn, Cheshire), using gradient elution. Eluent A consisted of 1M ammonium acetate, pH 5.16/acetonitrile (9:1 v/v) and B consisted of methanol/acetonitrile (9:1 v/v). Using a flow rate of 1ml/min, a linear gradient of 45%-90% B was run over a period of 20 min, commencing at the time of sample injection. 90% B was maintained for a further 5 min prior to re-equilibration over a linear, three minute gradient. Samples and standard solutions (20-200μl) were injected via a Rheodyne (Cotati, CA, USA) 7125 injector fitted with a 500μl loop.

5.2.2 Sample preparation and quantitation

Blood from balb/C mice injected with CLB-SPD or chlorambucil was mixed with potassium EDTA in eppendorf tubes, cooled on ice and centrifuged (1000rpm, 10 min, 4°C). The plasma obtained (200μl) was removed and mixed with 100μl TCA (25%)/DMSO (4:1 v/v) containing the internal standard p-iodoaniline (5 or 10μg/ml). Samples were cooled on ice for 5 min and the acid precipitate formed pelleted by centrifugation (3000rpm, 10min, 4°C). The supernatant solution was collected and stored at -20°C prior to analysis.

Body tissues, including implanted tumours, were homogenized in 0.9% (w/v) NaCl at 4°C and 200μl homogenate mixed with 100μl TCA (25%)/DMSO (4:1 v/v) containing p-iodoaniline (2μg/ml). The acid precipitate formed was pelleted as above and the samples analysed by HPLC using

the conditions described.

For the *in vitro* uptake studies, 15 - 20x10^6
ADJ/PC6 cells were incubated with CLB-SPD or chlorambucil
in a volume of 20ml. Following the incubations, excess
cold spermidine (100μM) was added to the cells to prevent
further uptake of CLB-SPD and the cells pelleted
(1000rpm, 10min). The cell pellet was washed three times
with 0.9% NaCl (w/v) (10ml) containing 100μM spermidine
to remove non-specifically bound CLB-SPD. The final cell
pellet was resuspended in 200μl 0.9% (w/v) NaCl and 100μl
extraction solvent containing p-iodoaniline (2μg/ml)
added. The precipitate formed was pelleted as before and
the supernatant analysed by HPLC.

CLB-SPD and chlorambucil were quantitated by peak
height. Calibration standards covering the range of 3 -
250ng CLB-SPD and 0.5 - 50ng chlorambucil were prepared
in 2 parts 0.9% NaCl and 1 part of the extraction solvent
and were injected onto the column. Retention times
relative to the internal standard p-iodoaniline were used
to identify peaks. Small portions of the standards were
stored frozen (-20^{0}C) until needed for analysis.

5.2.3 Recoveries

Plasma and tumour tissue homogenates were spiked
with CLB-SPD (500ng/ml and 5000ng/ml) and chlorambucil
(100ng/ml and 1000ng/ml) and extracted as described
above. These were compared in a direct assay to
standards in NaCl.

5.3 Results

5.3.1 Separation, quantitation and recoveries of CLB-SPD and chlorambucil

The HPLC method described gave a good separation of CLB-SPD, chlorambucil and the internal standard, p-iodoaniline with retention times of 12, 16 and 8.8 minutes respectively. Fig 5.1 shows the chromatographic profiles of these compounds. No coeluting interfering peaks were found in blank plasma or tissue extracts (fig 5.2a and 5.2b).

Absolute peak heights rather than those relative to p-iodoaniline were used for calibration due to the elution of p-iodoaniline on the solvent front of some tissue extracts. The calibration curves for CLB-SPD (3 to 250 ng injected) and chlorambucil (0.5 to 50 ng injected) were linear with correlation efficients of 0.999 and 0.996 respectively. Minimum detectable levels for CLB-SPD and chlorambucil were 3ng and 0.5ng injected respectively. Absolute recoveries from spiked plasma and tumour homogenate are shown in table 5.1 and were approximately 90% for CLB-SPD and 60% for chlorambucil.

Preliminary experiments in which tissues and plasma from mice injected with CLB-SPD and chlorambucil were analysed using the method described were performed and the method deemed suitable for use in further metabolism and distribution studies. However, in order to obtain the most reproducible results, it was found to be essential to use the highest possible quality reagents at all stages of the procedures described. In addition, an equilibration time of 15 minutes, at the initial solvent conditions, was also optimal.

Fig 5.1 **Chromatographic profiles of chlorambucil, CLB-SPD, and p-iodoaniline**
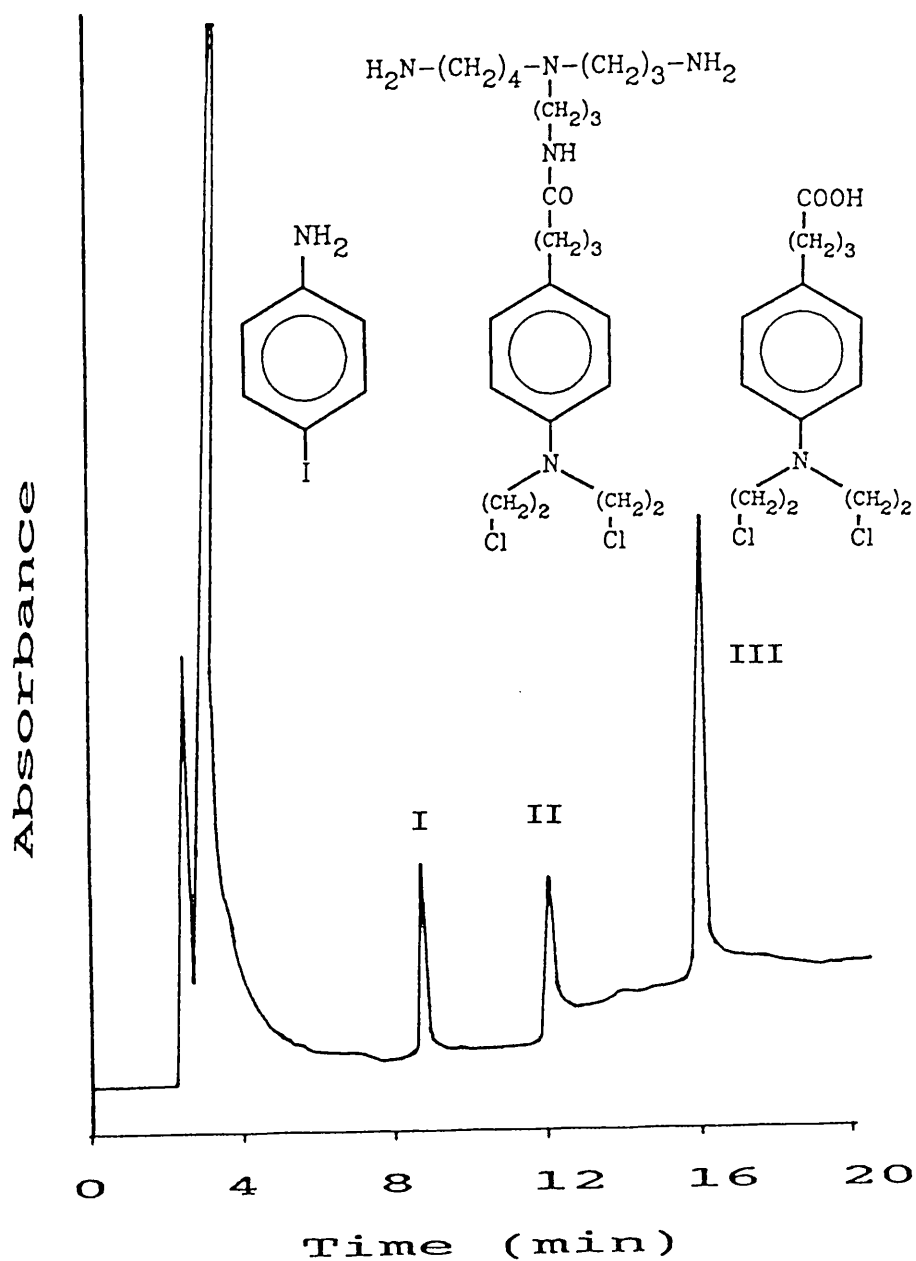
Gradient HPLC chromatogram of a TCA/DMSO extract of a mixed standard. Peak I: p-iodoaniline (internal standard), peak II: CLB-SPD, Peak III: chlorambucil

149

Fig 5.2 HPLC chromatograms of plasma and ADJ/PC6 tumour
extracts following CLB-SPD and chlorambucil
administration to mice.
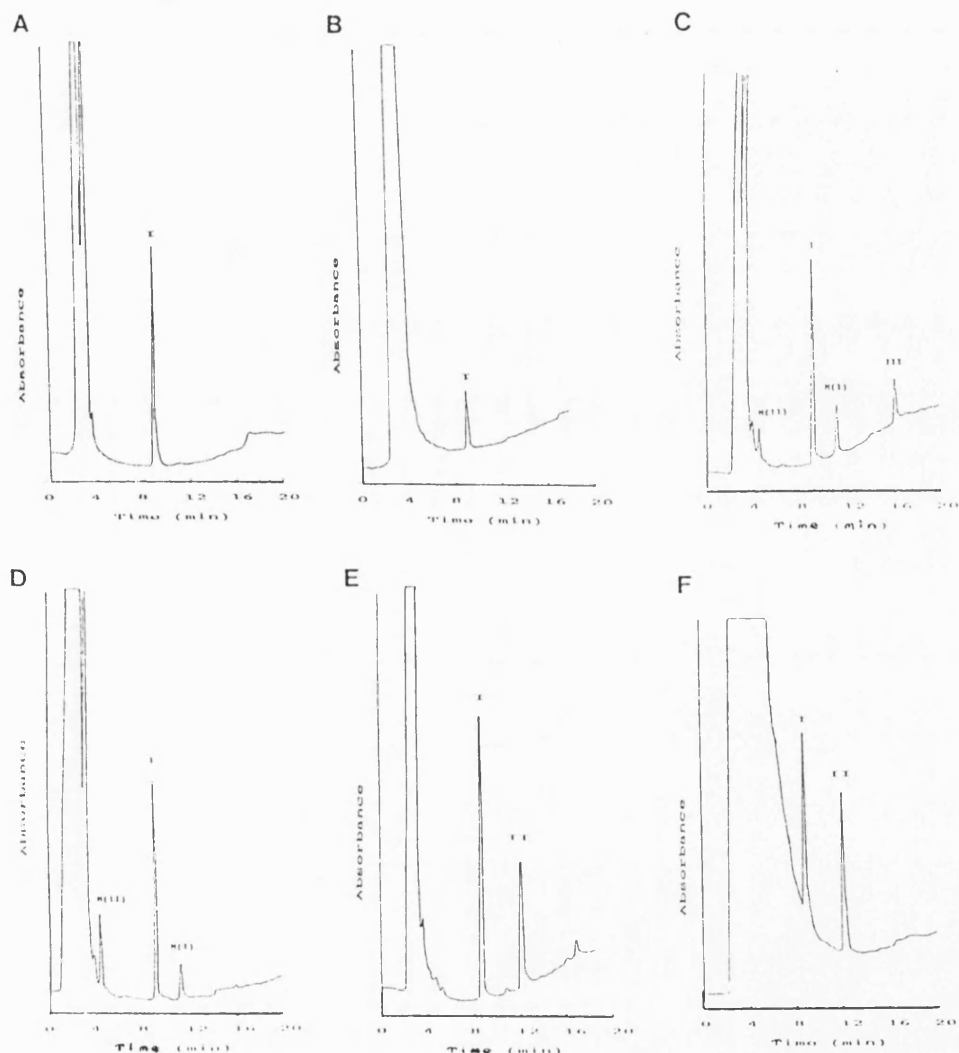
Mice were injected with chlorambucil (1.9mg/kg), CLB-SPD
(4mg/kg) or vehicle alone. Plasma and tumour extracts
were analysed by HPLC. The chromatograms shown
correspond to:
a) blank plasma extract (20μl inj.)
b) blank tumour extract (20μl inj.)
c) plasma extract, t=10 min following chlorambucil
administration (20μl inj.)
d) plasma extract, t=90 min following chlorambucil
administration (20μl inj.)
e) plasma extract, t=20 min following CLB-SPD
administration (30μl inj.)
f) tumour extract, t=75 min following CLB-SPD
administration (200μl inj.)

150

Table 5.1 Percentage recoveries of CLB-SPD and chlorambucil from tissue homogenates and plasma.

compound	concentration µg/ml	% Recovery (mean ± S.D.)	
		tumour tissue	plasma
chlorambucil −spermidine	5.0	96.7 ± 3.7	83.8 ± 1.5
	0.5	91.7 ± 3.2	85.1 ± 4.8
chlorambucil	1.0	64.9 ± 2.6	58.7 ± 0.98
	0.1	54.2 ± 3.4	61.1 ± 1.16

Mouse tissue homogenates and plasma were spiked with CLB-SPD and chlorambucil at the concentrations shown. TCA/DMSO extracts were analysed by HPLC and levels compared to spiked 0.9 % NaCl (w/v) controls. Values shown represent the mean percentage recovery ± S.D. (n=5).

5.3.2 Plasma levels of CLB-SPD and chlorambucil in mice following i.p. injection

Fig 5.3 shows the plasma levels of CLB-SPD and chlorambucil at various time intervals following the i.p. injection of equimolar doses (6.3μmol/kg) of the compounds (4mg/kg and 1.9mg/kg) respectively. The doses were chosen since 4mg/kg CLB-SPD was active against the ADJ/PC6 plasmacytoma in chapter 4. Plasma levels of CLB-SPD were approximately four fold greater than chlorambucil. Plasma clearance of both compounds appeared to be biphasic. Chlorambucil was cleared more rapidly over the first 30 min ($t_{1/2}$(chlorambucil) = 7 min, $t_{1/2}$(CLB-SPD) = 22 min) and CLB-SPD more rapidly after 30 min ($t_{1/2}$(CLB)= 15 min, $t_{1/2}$(CLB-SPD)= 7 min. Overall the rate of plasma clearance was approximately the same for both compounds. Due to the higher initial plasma levels, CLB-SPD could be detected upto 90 min in the plasma whereas chlorambucil was only detectable for 60 min.

Following chlorambucil administration two additional peaks, MI (Rt= 10.8 min) and MII (Rt= 4.2 min) were obtained on the HPLC chromatograms (fig 5.2c and 5.2d). These were possible metabolites of chlorambucil. Fig 5.4 shows the time course of their appearance and their removal from plasma . The metabolites were still detectable 3 hours following chlorambucil administration to mice. Based on their plasma half lives (MI $t_{1/2}$ = 65 min, MII $t_{1/2}$ = 45 min) which were measured 2 hrs after chlorambucil administration, and their order of appearance in the plasma (fig 5.4), it was deduced that they were possibly phenylacetic acid mustard (PAAM) (MI) and dechlorophenylacetic acid mustard (DeC-PAAM) (MII). Due to the lack of standards for these compounds they were quantitated on the basis that their molar absorption coefficients should be similar to chlorambucil.

Fig 5.3 Plasma levels of CLB-SPD and chlorambucil in mice.
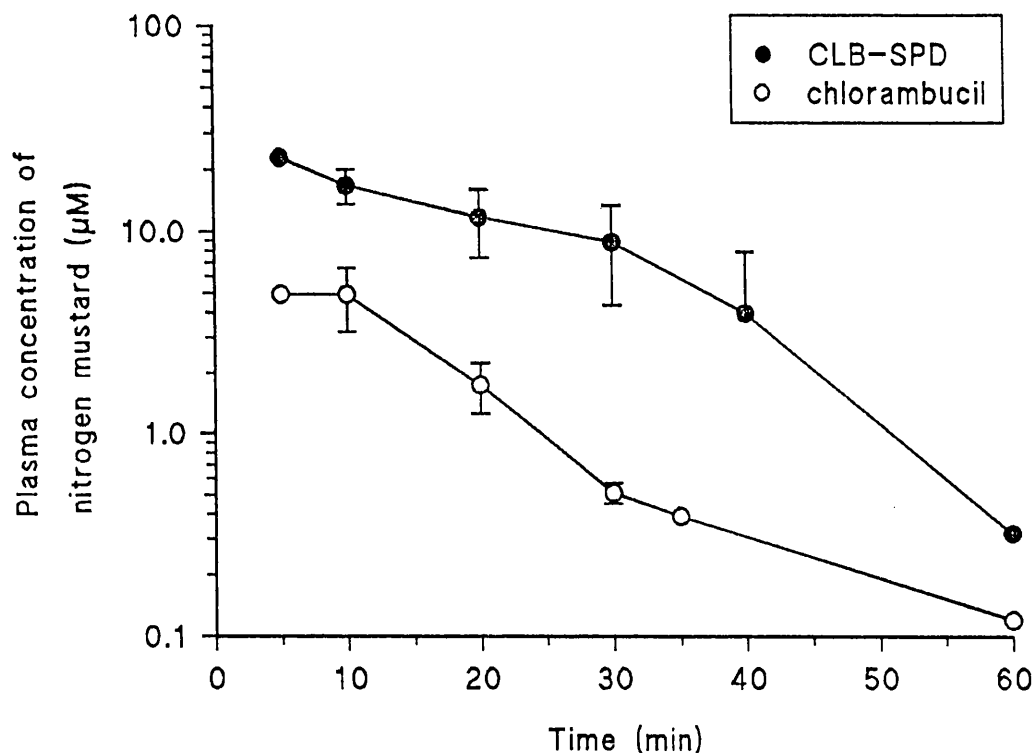
Mice were dosed with a single, equimolar (6.3μmoles/kg) i.p. injection of CLB-SPD (4mg/kg)(●) or chlorambucil (1.9mg/kg)(O) and plasma levels of the compounds at various time intervals measured by HPLC of TCA/DMSO extracts. Values shown are the mean ± S.D. of at least 3 mice or where error bars are not shown, 2 mice.

Fig 5.4 Plasma levels of two chlorambucil metabolites following chlorambucil administration.
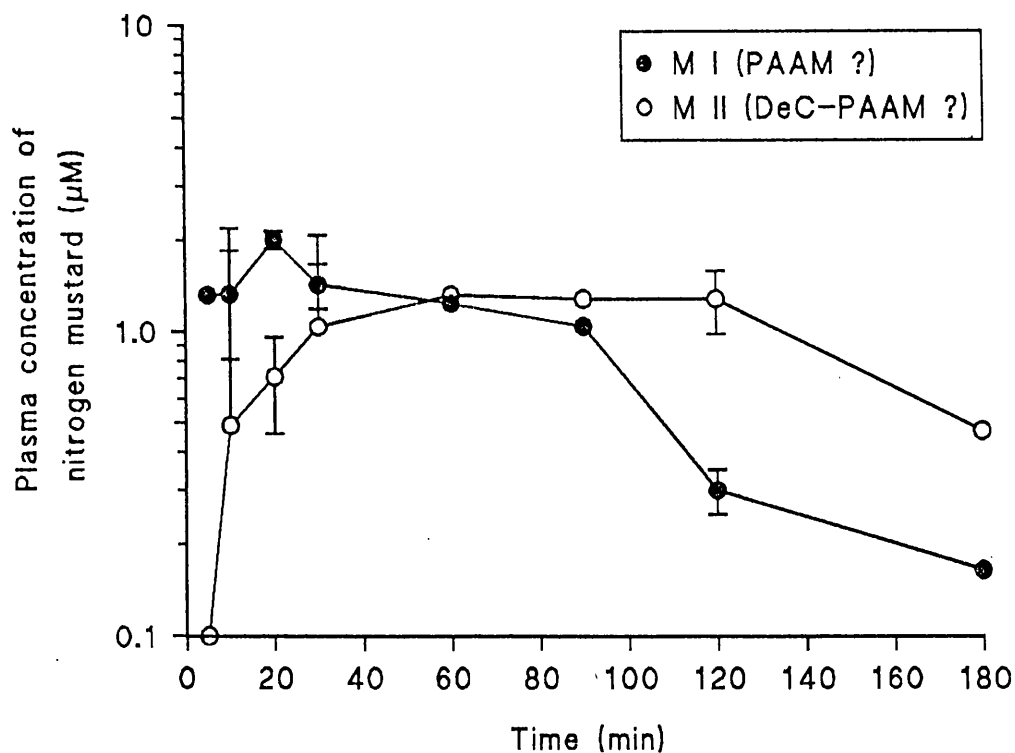
Mice were administered a single i.p. dose of chlorambucil (6.3 μmoles/kg) and the plasma levels of two metabolites MI (PAAM?) ($\bullet$) and MII (DeC-PAAM?) (O) measured by HPLC of TCA/DMSO extracts. Data shown are the means $\pm$ SD of at least 3 mice except where error bars are not shown and n=2.

Neither chlorambucil nor these chlorambucil
metabolites nor any other unidentified peaks were
detected in plasma after CLB-SPD administration (fig
5.2e).

5.3.3 Comparison of the *in vitro* uptake of CLB-SPD and chlorambucil by ADJ/PC6 cells

The uptake of CLB-SPD by ADJ/PC6 cells was
greater at 37°C than 4°C (Fig 5.5). Levels of CLB-SPD
achieved in ADJ/PC6 cells following a 60 minute
incubation at 37°C were also approximately 20 fold greater
than those obtained for chlorambucil under identical
conditions (Fig 5.5).

Further studies demonstrated that CLB-SPD uptake
was saturable in ADJ/PC6 cells with Km = 12.8μM and Vmax
= 0.31 pmoles/min/10^6 cells (Fig 5.6).

5.3.4 Comparison of tumour tissues levels of CLB-SPD and chlorambucil

The tissue levels of CLB-SPD and chlorambucil
obtained following i.p. injection of mice with equimolar
(6.3μmoles/kg) doses of the compounds (4mg/kg and 1.9
mg/kg respectively) were investigated. Fig 5.7 shows the
tumour tissue levels obtained. The maximum concentrations
of CLB-SPD achieved were approximately 10-fold greater
than chlorambucil. Maximum tumour tissue levels of
chlorambucil were seen 5 minutes following
administration. The levels dropped rapidly and could be
detected for no longer than 1 hour after administration
(fig 5.7). However, a metabolite (Rt=10.8 min), possibly
PAAM (MI), was detected even after chlorambucil had
disappeared   . DeC-PAAM (Rt=4.2 min)
was not detected since it eluted on the solvent front of
tissue extracts.

Fig 5.5 The *in vitro* uptake of CLB-SPD and chlorambucil into ADJ/PC6 cells; the effect of temperature.
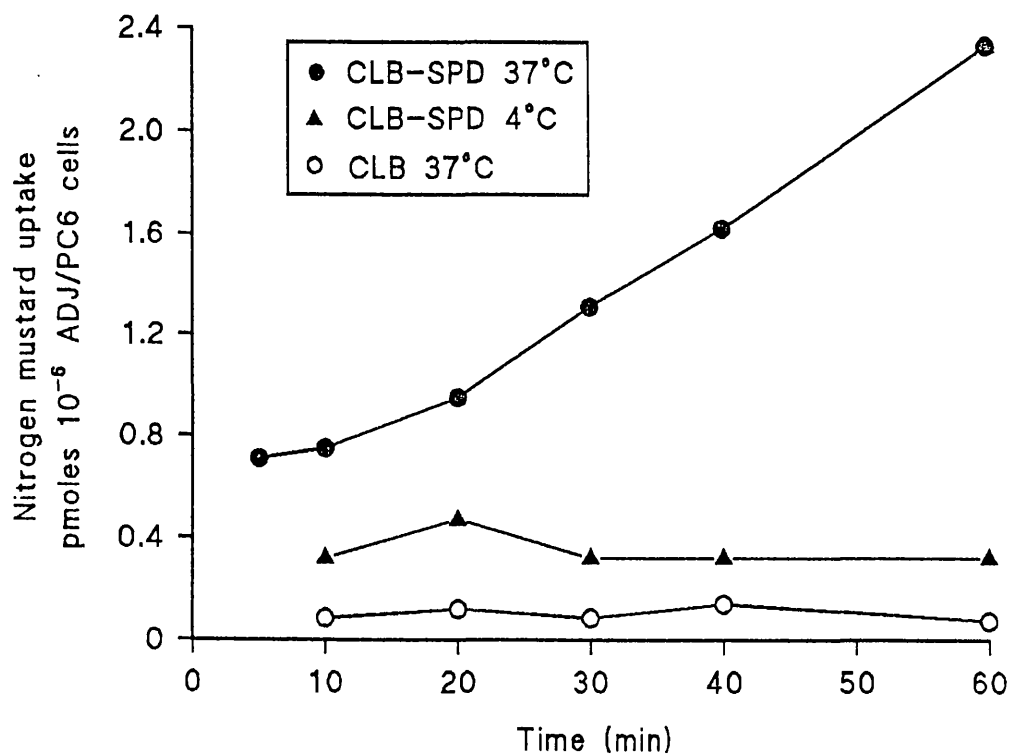
ADJ/PC6 cells (20 x 10^6) were incubated with CLB-SPD (2.5μM) at 37°C and 4°C or chlorambucil (2.5μM) at 37°C for 0-60 min. Cells were washed and TCA/DMSO cell extracts were analysed by HPLC. Data are the mean levels obtained in 2 experiments, both performed in duplicate.

Fig 5.6   The *in vitro* uptake of CLB-SPD into ADJ/PC6 cells; the effect of CLB-SPD concentration.

A

B
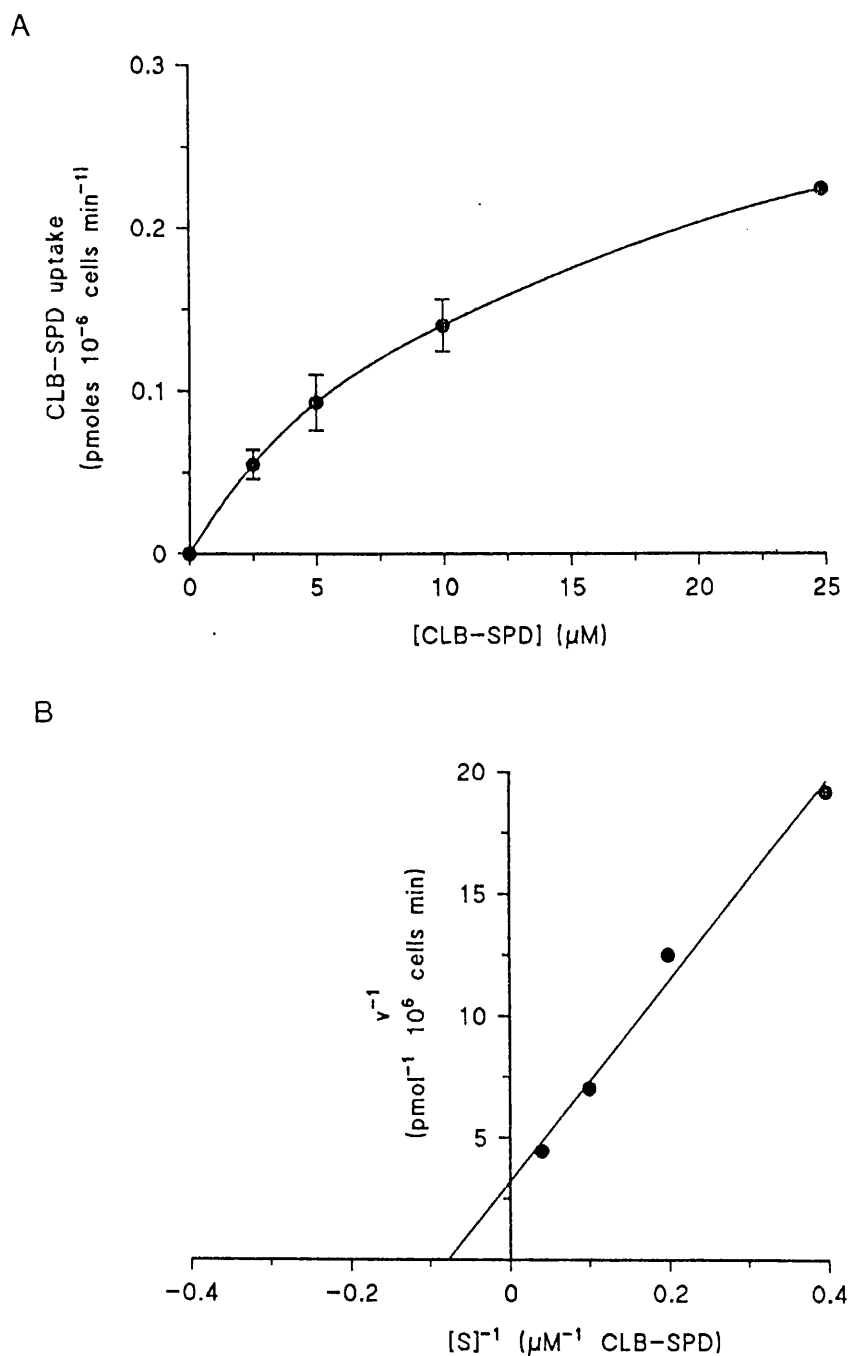
ADJ/PC6 cells (20 x 10^6) were incubated with CLB-SPD (2.5 - 25μM) for 60 min at 37°C.   TCA/DMSO extracts of cells were analysed by HPLC to determine CLB-SPD levels. Results are expressed as a linear rate plot (a) and a Lineweaver-Burk plot (b) from which the Km and Vmax values were obtained. Data represent the mean ± S.D. of at least 3 experiments except where no error bar is shown and n = 2.

Fig 5.7 Comparison of tumour tissue levels of CLB-SPD conjugate, chlorambucil and a chlorambucil metabolite following i.p. administration of CLB-SPD and chlorambucil.
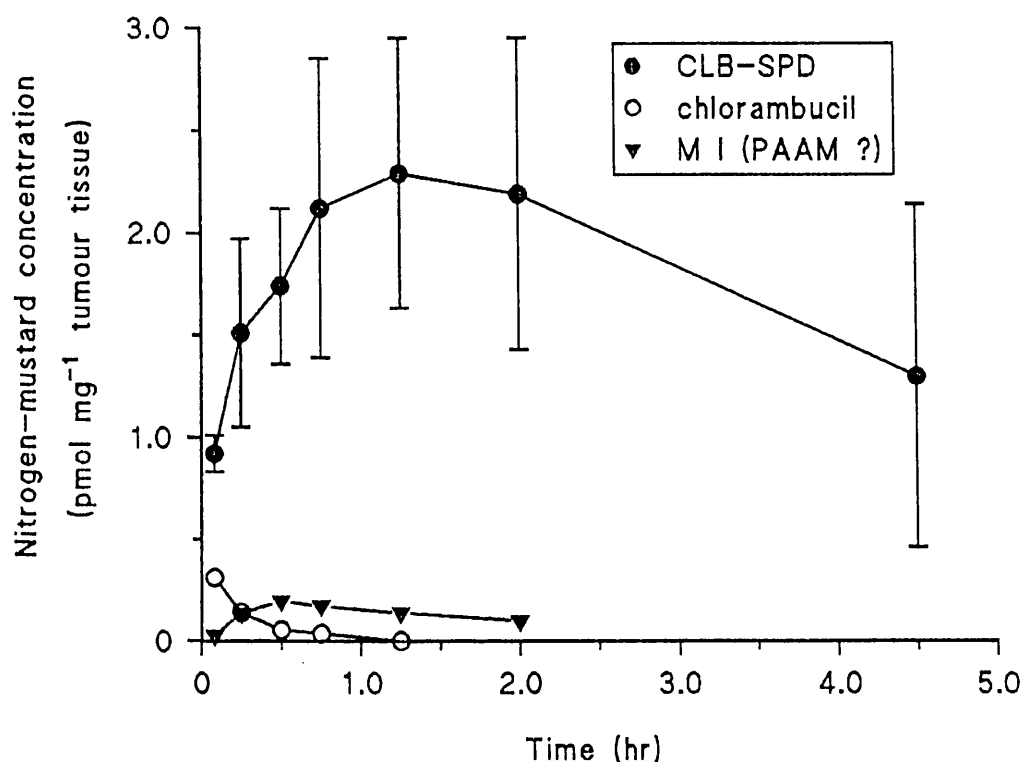
Mice innoculated with the ADJ/PC6 tumour were administered a single i.p. dose of CLB-SPD conjugate or chlorambucil (6.3 μmoles/kg) and sacrificed at various time intervals. Tumour tissue levels of CLB-SPD (●),chlorambucil (○) and chlorambucil metabolite MI (PAAM?)(▼) by HPLC analysis of TCA/DMSO extracts of tumour homogenates. CLB-SPD data represent the means ± S.D. of levels in tumour tissue from at least three mice per time point. For chlorambucil and chlorambucil metabolite (MI), data represent the mean levels in tumour tissue from two mice per time point.

In contrast to chlorambucil, for CLB-SPD a gradual increase in tumour tissue levels was seen with maximum levels achieved about 1 hour after administration. High levels of CLB-SPD were maintained for at least 4 hours and suprisingly, could still be detected 24 hr after administration. Mass spectrometry of this eluted fraction showed that this was unchanged CLB-SPD (Fig 5.8). No chlorambucil or its metabolites MI and MII were detected in any tumour tissue extracts (fig 5.2f).

5.3.5 Comparison of the tissue distribution of CLB-SPD and chlorambucil

Figs 5.9 and 5.10 show the concentrations of CLB-SPD and chlorambucil in a variety of tissues following the i.p. administration of equimolar doses of the compounds as in 5.3.4. In general, higher concentrations of CLB-SPD than chlorambucil were seen in all tissues. Particularly striking were the very high levels of CLB-SPD found in kidney and liver tissue which remained high for 24 hrs (Fig 5.9). Chlorambucil also achieved its highest levels in these tissues but did not remain elevated (fig 5.10) Mass spectrometry of the eluted CLB-SPD peaks of kidney extracts showed that this was unchanged CLB-SPD (Fig 5.11).

Chlorambucil was cleared rapidly from all tissues and was not detectable in any tissue for longer than 2 hours after administration (Fig 5.10). In contrast, CLB-SPD could still be detected in all tissues studied 24 hours after administration (Fig 5.9).

Neither chlorambucil nor its metabolites were detected in any tissue extracts following CLB-SPD administration (results not shown).

Fig 5.8 Mass spectra confirming the presence of CLB-SPD in tumour tissue 24hr after administration
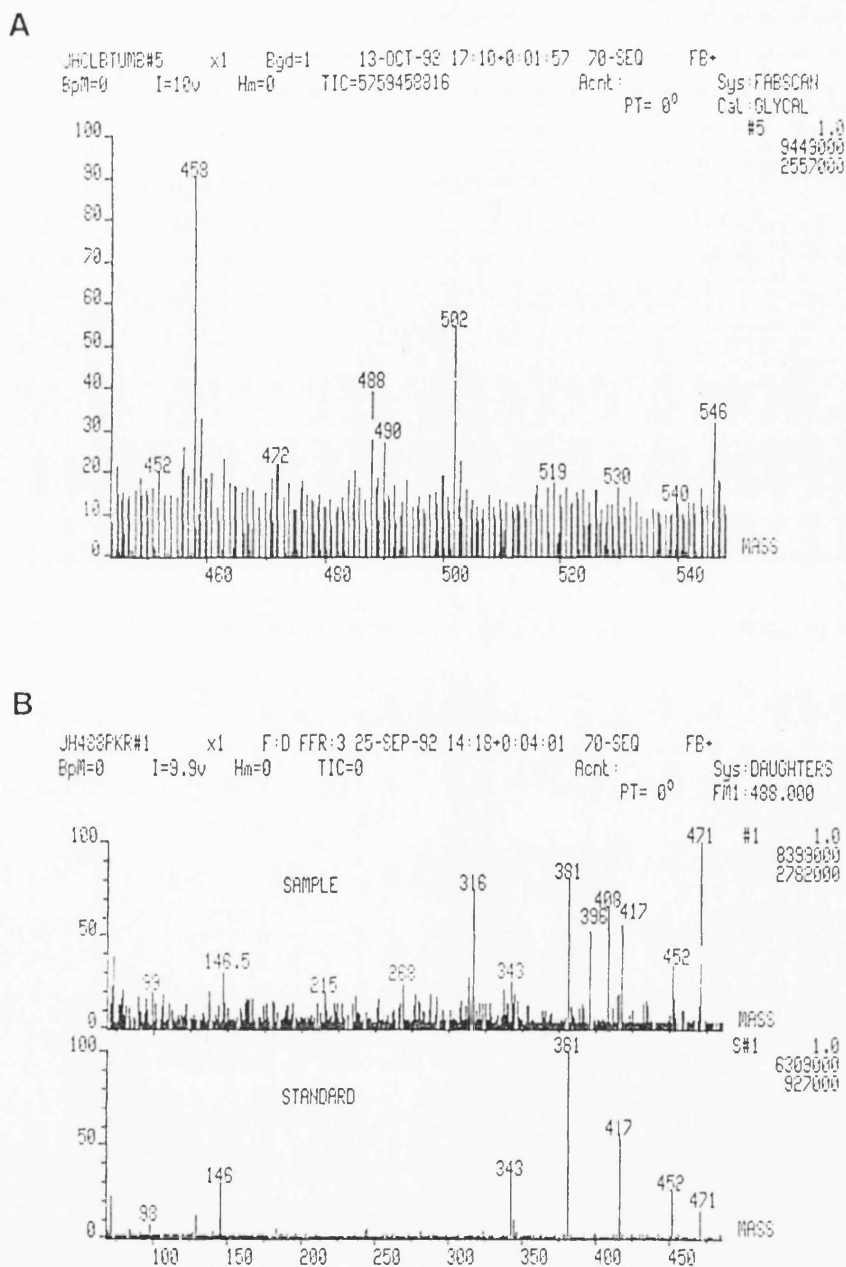
ADJ/PC6 tumour tissue was analysed by HPLC 24hr after CLB-SPD (4mg/kg) administration. The eluted fraction corresponding to CLB-SPD (Rt=12min) was collected, evaporated to dryness under nitrogen and analysed by mass spectrometry. The mass spectrum obtained is shown (a). For further confirmation of identity, MS-MS of the molecular ion corresponding to CLB-SPD ($M^+=488$) of the eluted fraction was performed and the spectra obtained compared to CLB-SPD standard (b).

Fig 5.9 Tissue distribution of CLB-SPD conjugate following i.p. administration.
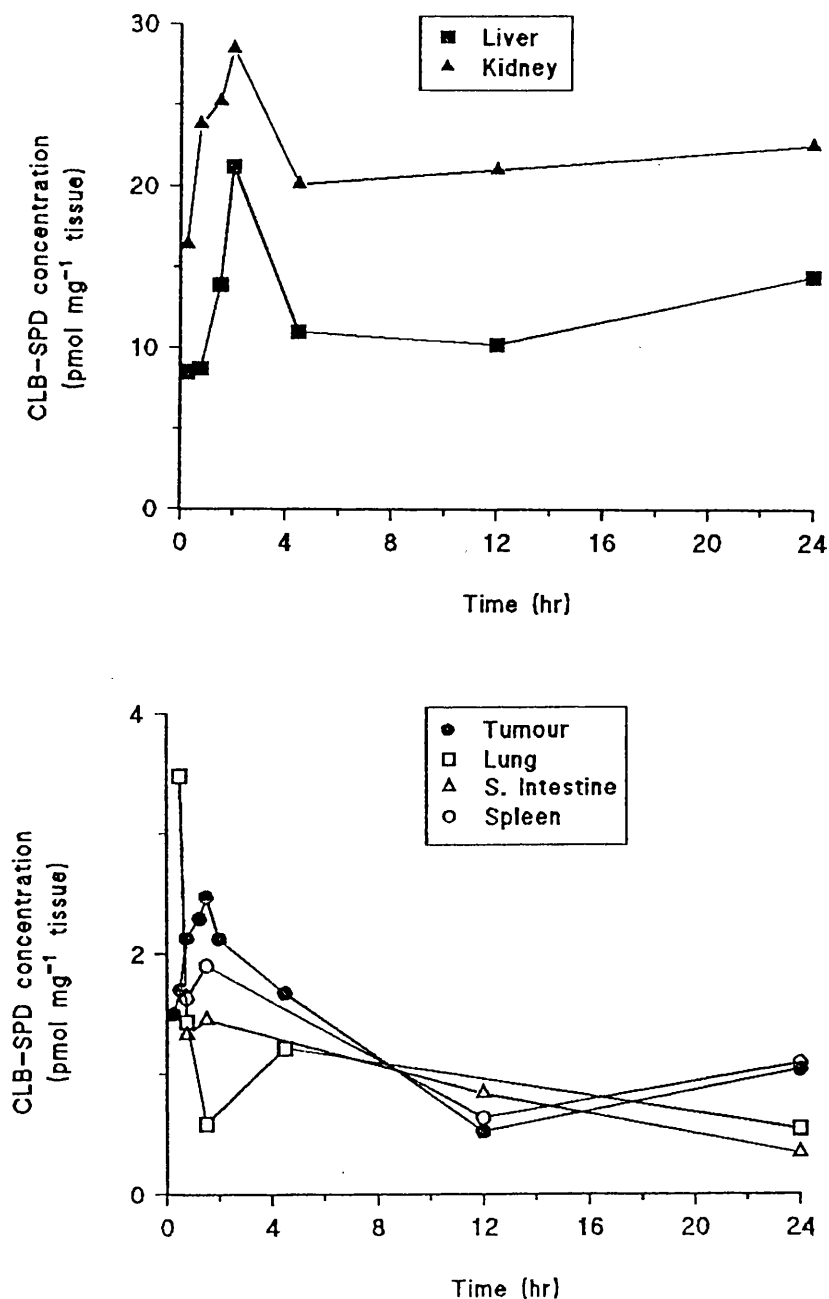
Mice innoculated with ADJ/PC6 plasmacytoma were administered a single i.p. dose of CLB-SPD conjugate (4mg/kg). The level of CLB-SPD in each tissue was determined at various time intervals by HPLC analysis of TCA/DMSO extracts of tissue homogenates. Data are representative of the means $\pm$ S.D. where n $\geq$ 3 except where error bars are not shown and n = 2.

161

Fig 5.10 Tissue distribution of chlorambucil following i.p. administration.
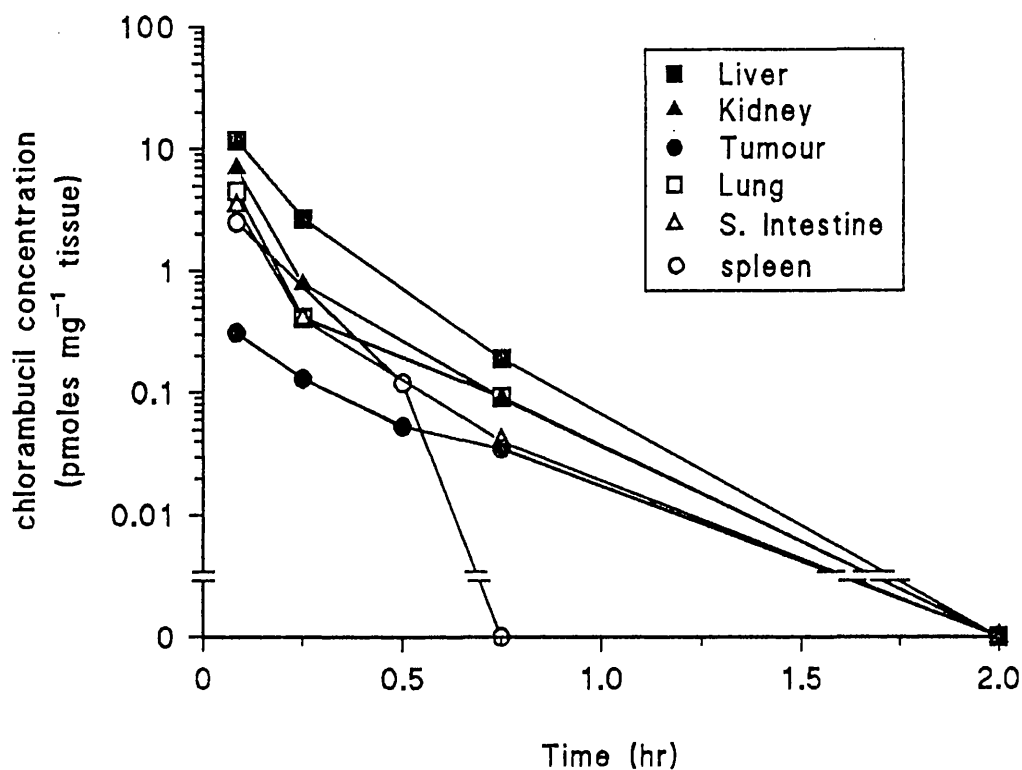
Mice innoculated with ADJ/PC6 plasmacytoma were administered a single i.p. dose of chlorambucil (1.9 mg/kg). The levels of chlorambucil in each tissue at various times were determined by HPLC analysis of TCA/DMSO extracts of tissue homogenates. Data are representative of the means of tissue from two mice per time point.

Fig 5.11 Mass spectra confirming the presence of CLB-SPD in kidney tissue 24hr after administration
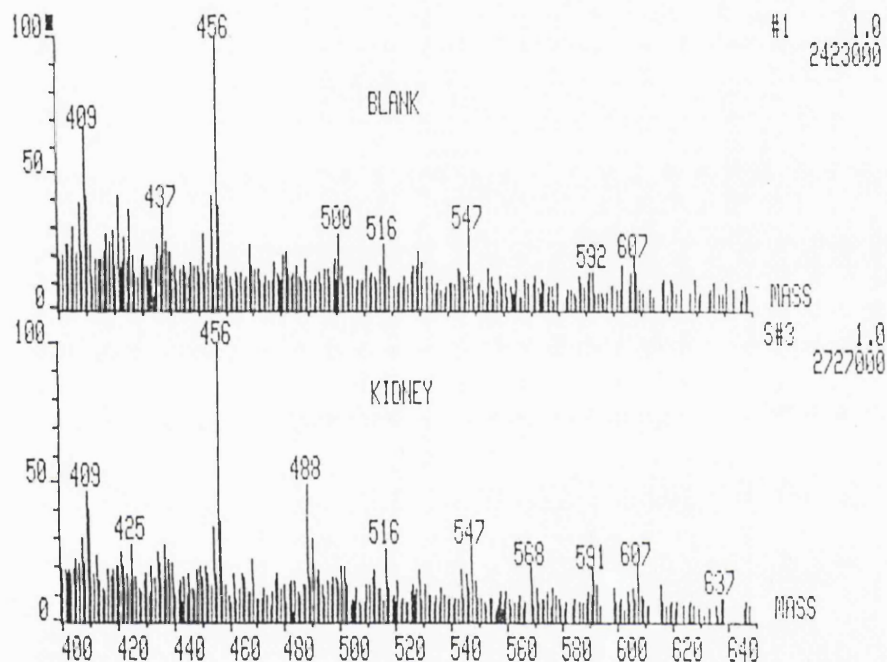
24hr following CLB-SPD (4mg/kg) administration, kidney extracts were analysed by HPLC and the eluted fraction corresponding to CLB-SPD (Rt=12min) evaporated to dryness and analysed by mass spectrometry. Mass spectra of the Kidney CLB-SPD fraction and a control fraction obtained on a blank run are shown.

5.4 Discussion

Previous *in vitro* studies with CLB-SPD conjugate demonstrated a 30-fold enhanced toxicity to ADJ/PC6 cells compared to chlorambucil, 10,000-fold enhanced DNA reactivity and a high affinity for the polyamine uptake system. However, *in vivo*, these results could not be fully reproduced and only a 2-fold increase in activity compared to chlorambucil was observed (chapter 4). There were several potential reasons for this, including the possibility of rapid *in vivo* metabolism of CLB-SPD by cleavage of the amide linkage to release free chlorambucil. In addition, the effectiveness of polyamine uptake as a delivery system for drug-conjugates was not known, since previously the potential uptake of CLB-SPD had been measured only indirectly by measuring its ability to inhibit ^{14}C-spermidine uptake. In fact, it is reported that the ability of antimalarial benzyl-polyamine analogues to inhibit polyamine uptake into human erythrocytes is not directly related to their accumulation (117). Compounds which were the best inhibitors of uptake were poorly accumulated and it was suggested that different systems existed for the uptake of endogenous polyamines and polyamine analogues in erythrocytes. The apparent high affinity of CLB-SPD for the polyamine uptake system is therefore not necessarily indicative that accumulation of CLB-SPD actually occurs. It was therefore not known whether increased delivery of the CLB-SPD conjugate compared to chlorambucil occured *in vitro* or *in vivo*, or whether the increased activity was simply the result of increased DNA reactivity.

To answer these questions, an HPLC method for the determination of CLB-SPD and chlorambucil in biological samples was required. The HPLC method developed was suitable for measuring tissue and plasma levels of CLB-SPD and chlorambucil following their administration to mice, as well as cellular levels achieved *in vitro*. A

previously reported method for chlorambucil analysis
(211) was unsuitable for CLB-SPD due to peak tailing.
Existing methods used for polyamine analysis were also
unsuitable. The gradient elution method described here
eliminated peak tailing and pre- or post-column
derivatization was unnecessary to detect the compounds.
Limits of detection were of a similar order of magnitude
to previously reported methods for chlorambucil analysis
(211,296). It should be stressed, however, that the
success of the method depended on the use of the highest
possible quality solvents and chemicals as well as
critical timing of the equilibration period. A major
drawback of the method was the long run time (45 minutes
total) and the lack of automation.

Recoveries of CLB-SPD (approximately 90%) were
very favourable and although chlorambucil recovery was
lower (approximately 60%) the method was deemed suitable
since the absolute concentrations of the compounds could
be obtained from their percentage recoveries.

Quantitation was performed using absolute peak
heights relative to linear standards. Peak heights
relative to the internal standard were not used since in
some cases the internal standard eluted on the shoulder
of the solvent front which gave variations in its peak
height and subsequent quantitation inaccuracies.

In vivo antitumour studies demonstrated only a 2-
fold increase in activity of the CLB-SPD conjugate
compared to chlorambucil (chapter 4). Initial studies
were carried out to determine whether this was due to
metabolism of the conjugate. The observation that no
chlorambucil was detected in any tissue or plasma samples
following CLB-SPD administration suggests that metabolism
of the conjugate by cleavage of the amide bond to give
free chlorambucil did not occur.

Following i.p. administration to mice, plasma
concentrations of CLB-SPD were 4-fold higher than
chlorambucil (fig 5.3). The differences in plasma

concentrations may either reflect differences in absorption from the peritoneal cavity or the differential abilities of chlorambucil and the CLB-SPD conjugate to be transported by red blood cells, a phenomenon reported for both alkylating agents (220) and polyamines (110). Drugs bound to red blood cells would not be detected using this method.

The plasma clearance of both compounds appeared to be biphasic,an observation made also by Lee *et al.* for chlorambucil (222,297). Overall, CLB-SPD was detected in the plasma longer than chlorambucil (90 mins compared to 60 mins) and therefore the area under the concentration versus time curve (ie bioavailability to tumour) was greater for the CLB-SPD conjugate than chlorambucil. However, if a large amount of chlorambucil was bound to red blood cells, it could be retained, undetected, in the blood for longer.

Two additional peaks (MI and MII, fig 5.3) were detected following chlorambucil administration and were possible chlorambucil metabolites. In mice, chlorambucil is metabolized extensively and rapidly by mitochondrial ß-oxidation of the butyric acid side chain to form phenylaceticacid (PAAM) via the intermediate dehydrochlorambucil (DeHCl) (fig 5.12). PAAM is then converted to the less active, monoalkylating species, dechlorophenylaceticacid (DeC-PAAM) which is the major post ß-oxidation metabolite (222). The two metabolites detected following chlorambucil administration may correspond to PAAM and DeC-PAAM based on the comparison of their respective half lives in this study of 65 and 45 minutes (Fig 5.4) to the literature values of 65 mins (PAAM) (222) and 40 mins (DeC-PAAM)(297). Other evidence for their identification is that the peak corresponding to PAAM was seen prior to that corresponding to DeC-PAAM (Fig 5.2 and fig 5.4). Standards of these compounds were not available for comparison.

Fig 5.12   The metabolism of chlorambucil by ß-oxidation
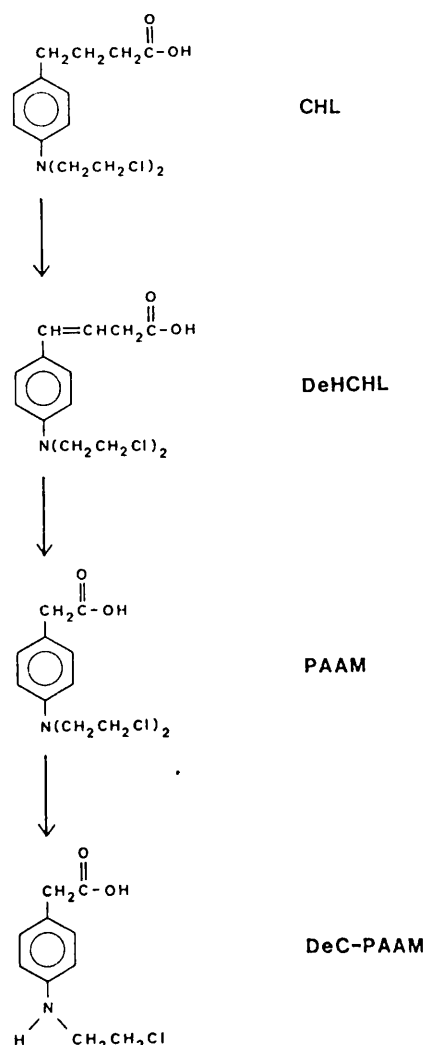
The chlorambucil metabolites had much longer plasma half lives than chlorambucil and were detectable in the plasma for upto 3 hours following chlorambucil administration (results not shown). Although non-bound chlorambucil was cleared rapidly, these active metabolites were present in the plasma and available for tissue uptake for a longer period than CLB-SPD.

The absence of the long lived chlorambucil metabolites in plasma or tissues from mice injected with CLB-SPD was further evidence in favour of the suggestion that CLB-SPD was not metabolised *in vivo* to chlorambucil

by cleavage of the amide bond. In addition, no other unidentified peaks were seen following CLB-SPD administration. This, together with the observation that unchanged CLB-SPD was detectable in many tissues three days after administration, suggested that little, if any, metabolism of the conjugate occurred. It therefore seemed unlikely that CLB-SPD metabolism, particularly cleavage of the amide bond, was the reason for its poor *in vivo* activity.

The observation that the amide bond is not cleaved suggests that CLB-SPD is not acting as a prodrug by releasing free chlorambucil. In fact, due to the apparent stability of the amide linkage, CLB-SPD appears to be a totally different chemical entity to chlorambucil. Since the carboxylic acid group required for ß-oxidation of chlorambucil metabolism is blocked in CLB-SPD, CLB-SPD cannot be metabolised by this route. This is an important observation since ß-oxidation plays an important role in the metabolism of chlorambucil in rats (223,224) and mice (222). It is reported that 40% of an administered dose of chlorambucil is metabolised via the ß-oxidation pathway (298). It is also reported that PAAM, which is a major product of chlorambucil ß-oxidation, has similar antitumour activity to chlorambucil but a lower therapeutic index due to greater general tissue toxicity (223). This could have important implications since if this metabolite is not formed from CLB-SPD, one may expect that CLB-SPD could demonstrate less general tissue toxicity than chlorambucil.

A potential route for the inactivation of CLB-SPD could be enzymic or non-enzymic conjugation to glutatione, which is a mechanism also used for chlorambucil inactivation (225,226,231). Hydrolysis by water could also occur. The degree of inactivation occurring via these routes would depend on the availability of GSH and GST-enzymes (226,231).

The uptake of CLB-SPD and chlorambucil in ADJ/PC6

168

plasmacytoma was compared *in vitro* and *in vivo*. *In vitro* CLB-SPD achieved a 20-fold higher concentration than chlorambucil in ADJ/PC6 cells (fig 5.5), which supports the suggestion that conjugation of chlorambucil to spermidine does increase its delivery to cells. CLB-SPD uptake was a saturable, temperature dependent process in ADJ/PC6 cells with Km= 12.8μM and Vmax= 0.3 pmoles/ min/10^6 cells (fig 5.6). These parameters show marked differences to those obtained for spermidine uptake in this cell line (Km=0.2μM, Vmax= 30 pmoles/min/10^6 cells) showing that CLB-SPD had both a lower affinity for uptake and a much reduced maximal velocity compared to spermidine. These results demonstrate that conjugation of chlorambucil to a polyamine carrier increases its accumulation into ADJ/PC6 cells *in vitro* although the affinity of CLB-SPD for the polyamine uptake system is relatively low. A draw back of the method used is that during sample processing, chlorambucil may diffuse back out of the cells (211) and the levels measured may not be representative of the true levels.

In vivo, maximal tumour levels of CLB-SPD were ten fold greater than those obtained for chlorambucil following i.p. administration of equimolar doses of the two compounds (fig 5.7). These results are of a similar order of magnitude to those obtained *in vitro* and are not subject to the diffusional errors associated with the *in vitro* studies. Therefore a higher accumulation of CLB-SPD than chlorambucil was apparent from *in vitro* and *in vivo* studies. In addition, tumour tissue levels of CLB-SPD increased gradually over the first hour after administration. This is indicative of some sort of gradual accumulation for CLB-SPD in contrast to chlorambucil which enters cells by diffusion (211), achieving maximal levels 5 minutes after administration and then declining rapidly at a similar rate to its disappearance from plasma.

Concentrations of CLB-SPD were greater than

chlorambucil in all tissues (fig 5.9 and 5.10). CLB-SPD
was also retained in tissues for several days compared to
chlorambucil which was rapidly removed. CLB-SPD levels
in kidney and liver were particularly high. Chlorambucil
itself also achieved its highest levels in liver and
kidney (fig 5.10) which is in agreement with Mitoma et
al. (299).

The increased accumulation of CLB-SPD into all
tissues suggests that its increased uptake was not
specific for tissues which had a particularly active
polyamine uptake system eg small intestine or tumour. A
similar lack of prostate tumour specificity is also
reported for the spermidine analogue, MGBG (73). However
an important observation was that whereas chlorambucil
levels were particularly low in tumour compared to other
tissues, conjugation to spermidine improved the delivery
to tumour compared to some of the other tissues eg small
intestine. In addition, although CLB-SPD concentrations
achieved in the tumour were not as great as hoped, the
tumour mass may have included a large proportion of dead
cells and connective tissue, which would not accumulate
the compound. Measurement of total tissue CLB-SPD did
not take into account these differences. Therefore,
uptake into live tumour cells may actually be higher than
it appears from these studies. A further possible reason
for lower than expected levels of CLB-SPD in tumour is
that the blood supply to certain parts of the tumour may
be poor.

Therefore both *in vitro* and *in vivo* studies
suggest that conjugation of chlorambucil to spermidine
results in enhanced delivery to cells compared to non-
conjugated parent compound. Enhanced uptake may be by
the polyamine uptake system alone or possibly by
facilitated diffusion as a result of the high binding
affinity of polyamines within the cell preventing the
compound diffusing back out of the cell. This may also
explain the differential uptake seen *in vitro* where

enhanced levels of CLB-SPD may have been due to chlorambucil but not CLB-SPD diffusing out of the cells on washing with saline. MGBG is also retained in cells in this manner (103,120,180). High intracellular binding would also explain why higher levels of CLB-SPD were seen in all tissues, not just those with a particularly active polyamine uptake system.

Based on the observations that CLB-SPD was not metabolised to chlorambucil and that higher tumour concentrations than chlorambucil are achieved, one would have expected much greater *in vivo* antitumour activity than was seen. This would seem plausable since a given concentration of the conjugate was much more reactive with naked DNA than chlorambucil. There are several possible explanations for poorer than expected *in vivo* activity. Firstly the DNA crosslinking studies were performed *in vitro* on naked DNA (291,300) in the absence of polyamines. In the cell, competition for DNA binding by endogenous polyamines and other cations such as Mg^{2+} would no doubt occur, reducing the DNA affinity and subsequent cross linking activity of the CLB-SPD conjugate. Secondly, the high affinity of polyamines for intracellular polyamine receptors, RNA, proteins and other anionic macromolecules may result in only a small proportion of intracellular CLB-SPD reaching the DNA. The fact that the CLB-SPD conjugate could be detected unchanged several days following administration suggests that it may become sequestered at sites distant from the DNA, possibly in a chemically unreactive form. One possibility is that the primary amine groups of the polyamine chain may shield the chloroethyl groups of chlorambucil portion of the conjugate, preventing both its metabolism and DNA alkylation. Alternatively, the chloroethyl groups may become embedded in the plasma membrane in a similar manner to chlorambucil in red blood cells (220). Intracellular binding may therefore reduce the toxicity of CLB-SPD to tumour cells. This is

contrast to MGBG, the toxicity of which is dependent on achieving high intracellular levels by receptor binding (103,120,131). The differences in sites of action of the two compounds may explain why sequestration is deleterious to CLB-SPD but not MGBG. In fact, if CLB-SPD cannot reach the DNA it may have an alternative mechanism of action. For example, it has been suggested that nitrogen mustard may inhibit membrane Na^+/K^+ ATPase in mouse ADJ/PC6 plasmacytoma cells (219).

A further point to consider is that for nitrogen mustards the site of DNA alkylation and crosslinking is the guanine N-7 position in the major groove (291,292). With CLB-SPD, interstrand cross linking may not necessarily be between guanine N-7 sites since initial sites for crosslinking will be determined by the spermidine moiety. The precise location and relative importance of these lesions to the cytotoxicity of the conjugate remain to be determined. The lesions themselves may be less lethal for CLB-SPD in that, for example, their repair may be easier.

In summary, it appears that the poor *in vivo* activity of CLB-SPD was not due to metabolism of the conjugate and cleavage of the amide linkage did not occur. In fact there appeared to be no metabolism of the conjugate whatsoever and it appeared to remain sequestered in tissues for many days. This sequestration may have prevented the conjugate reaching its DNA target. Enhanced delivery of CLB-SPD compared to chlorambucil occurred in ADJ/PC6 plasmacytoma both *in vitro* and *in vivo*. However, increased uptake was seen in all tissues and not just those with a particularly active polyamine uptake system such as small intestine and tumour. Using this method, however, it was not possible to detect differences in uptake in specific cell types within a tissue.

These results suggest that the increased accumulation of CLB-SPD compared to chlorambucil may not

be soley due to the polyamine uptake system and the role
of the polyamine uptake system in the delivery of the
conjugate is unclear. Instead, some kind of facilitated
diffusion may occur, with the CLB-SPD conjugate binding
to receptors within the cell, resulting in greater
accumulation than can be achieved for chlorambucil alone.

CHAPTER 6 - Comparison of the *in vivo* tissue distribution and tumour uptake of CLB-SPD and MGBG

6.1 Introduction

The tissue distribution of exogenously administered polyamines is reported to vary greatly in rodents and may be due to different physiological requirements of cells for the polyamines or different binding affinities of the different polyamines for intracellular substances (73,301). In chapter 5, the tissue distribution of CLB-SPD was investigated and high concentrations were found in the kidney and liver compared to other tissues following i.p. administration. In contrast, CLB-SPD levels in the small-intestine were low. This was suprising considering that the small intestine is reported to accumulate polyamines and the spermidine analogue MGBG (73,74,148,188,302) possibly via the polyamine uptake system. The role of the polyamine uptake system in the uptake of CLB-SPD was not clear.

In this chapter, the tissue distributions of CLB-SPD and MGBG were compared to determine whether the high kidney and liver concentrations obtained for CLB-SPD were peculiar to CLB-SPD or seen also with MGBG. In addition, the effect of *in vivo* polyamine depletion (with DFMO) on the tissue distribution of both compounds was investigated. Polyamine depletion is reported to increase the uptake of polyamines and polyamine analogues into rapidly proliferating tissues (74,146,148,188,303) and it was interesting to see whether DFMO had a similar effect on CLB-SPD uptake.

Attempts were also made to alter the tissue distribution of CLB-SPD and MGBG using osmotic minipumps and continuous i.v. infusion (CLB-SPD only) to deliver low concentrations of the compound over long time periods. The rationale behind this was that slow delivery of polyamine analogues at concentrations near

their Km for uptake should selectively enhance uptake
into tissues possessing a polyamine uptake system (267).
Increased delivery of CLB-SPD to ADJ/PC6 plasmacytoma
would be expected if the compound utilized the polyamine
uptake system, as well as to other tissues such as small
intestine and lung.

6.2 Results

6.2.1 Comparison of the tissue distribution of CLB-SPD and MGBG in mice

The tissue distributions of CLB-SPD and MGBG were
measured following the administration of a single i.p.
dose of 4mg/kg CLB-SPD (6.3μmoles/kg) or 40 mg/kg MGBG
(145μmoles/kg). In the CLB-SPD studies, mice were
innnoculated with ADJ/PC6 plasmacytoma and for the MGBG
studies the mice were innoculated with EATC. The
different tumours were used due to the unavailability of
ADJ/PC6 plasmacytoma when the MGBG studies were carried
out. Also, a higher dose of MGBG than CLB-SPD was
administered since this was similar to the therapeutic
dose used in man (188). The results were expressed as
pmoles compound/mg tissue per mmole/kg dose to allow a
direct comparison of tissue levels and distribution (fig
6.1). This was felt to be an appropriate way of
expressing the relative tissue distributions since it is
reported that altering the dose of polyamine given does
not alter its distribution pattern (73).

The most striking difference in the tissue
distributions of CLB-SPD (t=90min) and MGBG (t=2hr) was
that much higher concentrations of CLB-SPD than MGBG were
seen in the kidney (fig 6.1). In contrast, MGBG achieved
much higher levels than CLB-SPD in the small intestine
(fig 6.1). For both compounds the liver contained a high
proportion of the recovered dose (fig 6.1). Tissue

Fig 6.1 Comparison of CLB-SPD and MGBG tissue
distribution.

A

Tissue levels of CLB-SPD
(pmoles mg^{-1} tissue)

4000   3000   2000   1000   0

Liver

Kidney

Lung

Spleen

Small-
intestine

ADJ/PC6
Tumour

CLB-SPD (90min)
CLB-SPD (24hr)

B

Tissue levels of MGBG
(pmoles mg^{-1} tissue)

0   1000   2000   3000   4000

Liver

Kidney

Lung

Spleen

Small-
intestine

MGBG (2hr)
MGBG (24hr)
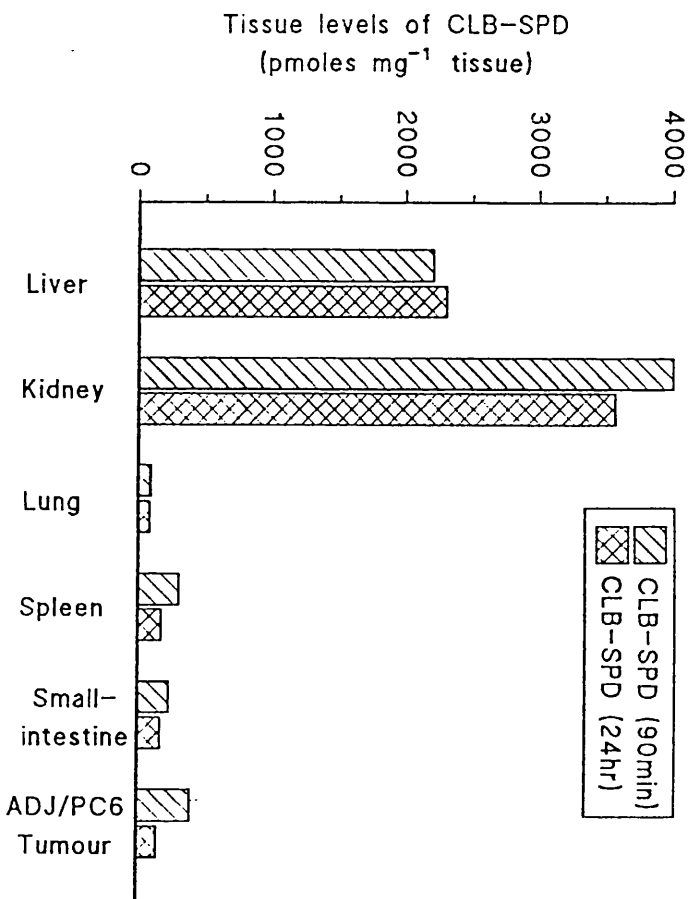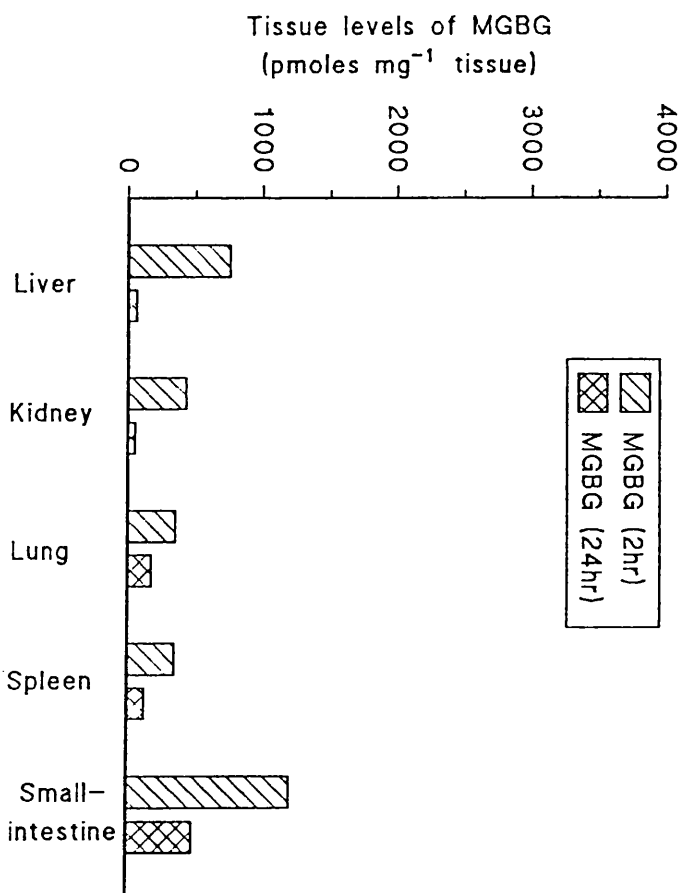
Legend to fig 6.1 Comparison of CLB-SPD and MGBG tissue distribution.

A single i.p. dose of CLB-SPD (4mg/kg) to mice innoculated with ADJ/PC6 plasmacytoma (a) or MGBG (40mg/kg) to mice innoculated with EATC (b), was administered. Tissue levels of CLB-SPD and MGBG were measured by HPLC analysis of TCA/DMSO and PCA tissue extracts respectively. Tissue levels obtained were expressed as concentration of drug per mmol/kg dose administered to allow direct comparison of the results.

MGBG data is the mean of tissues from 2 mice per time point and is representative of two experiments. CLB-SPD data represents the mean of tissues from 3 mice.

levels of CLB-SPD in spleen, small-intestine and ADJ/PC6 tumour (fig 6.1a) and of MGBG in the lung, spleen and kidney (fig 6.1b) were low and of a similar order of magnitude. However, the concentration of CLB-SPD in the lung was approximately 4-fold lower than the ADJ/PC6 tumour (fig 6.1a).

The concentrations of CLB-SPD and MGBG in ADJ/PC6 plasmacytoma and EATC respectively were compared. From fig 6.2 it can be seen that the maximum level of MGBG achieved in EATC was 1.3 nmole/10^6 cells. Since 1×10^6 EATC weighed 4mg (data not shown), this concentration is equivalent to 325pmoles/mg EATC. In contrast, the maximum level of CLB-SPD achieved in ADJ/PC6 plasmacytoma was 2.4 pmoles/mg tissue (fig 5.7) which was 135-fold lower than the MGBG concentration obtained in EATC. On a molar basis, the dose of MGBG administered, was approximately 20-fold greater than the CLB-SPD dose. Taking this into consideration, this means that for equivalent molar doses, MGBG reached an approximately 7-fold higher concentration in EATC than CLB-SPD did in the ADJ/PC6 tumour.

CLB-SPD also tended to be retained in most tissues for at least 24hr, in particular in the kidney, liver and lung (fig 6.1a). In contrast, MGBG was not retained in tissues to such an extent (fig 6.1b). Further investigation revealed that tissue concentrations of MGBG were relatively stable until t=6 hr, except in liver and small intestine where levels dropped suddenly after t=2 hr and then decreased at the same rate as the other tissues (fig 6.2a).

Fig 6.2 Tissue distribution of MGBG and its rate of removal from tissues and EATC.
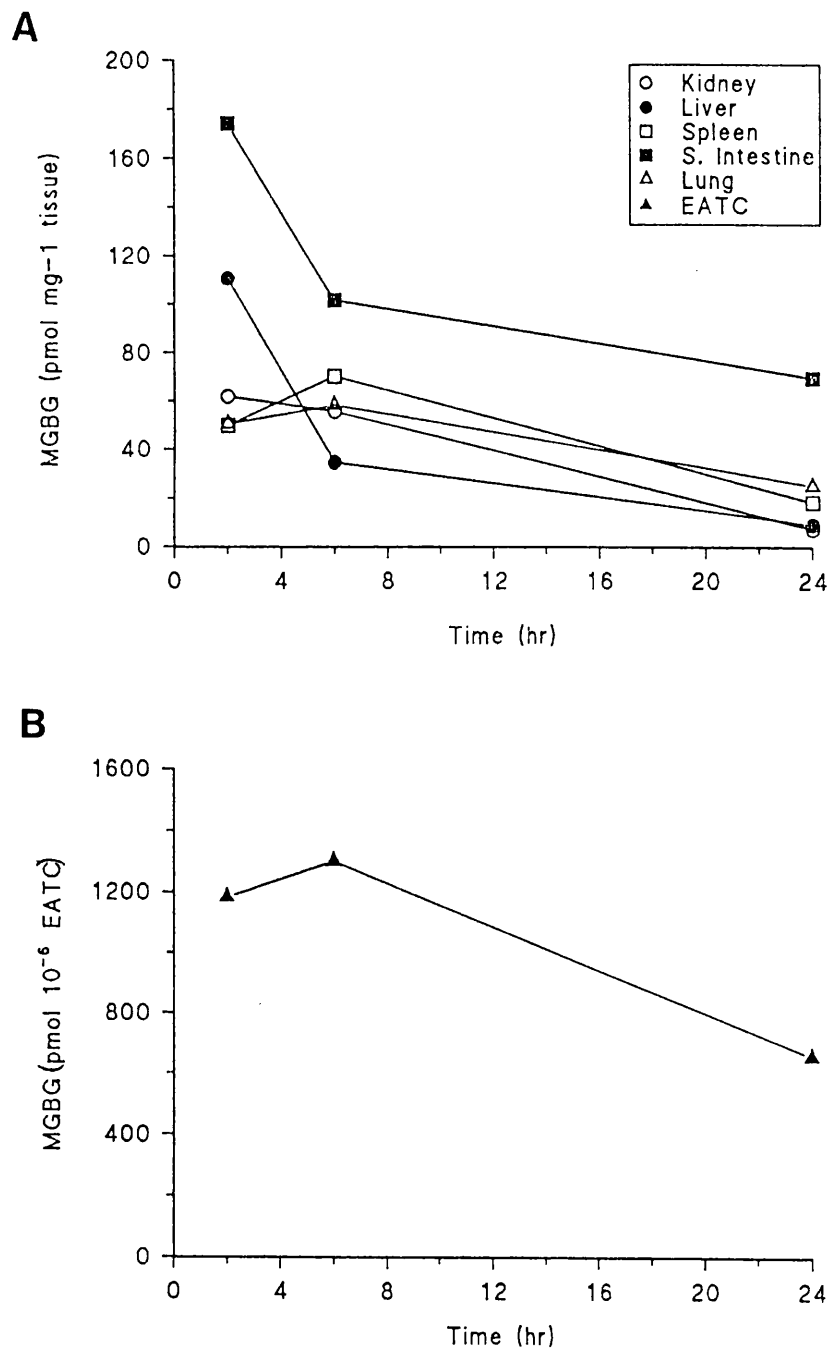
Mice, pre-innoculated with EATC, were administered a single i.p. dose of MGBG (40mg/kg). The concentration of MGBG in tissues (a) and EATC (b) was measured by HPLC analysis of PCA tissue and cell extracts at t=2, t=6 and t=24 hr. Data are the mean tissue levels for two animals per time point and are representative of two experiments.

6.2.2 The effect of *in vivo* polyamine depletion on the tissue distribution of CLB-SPD and MGBG

The effect of polyamine depletion on the tissue levels and distribution of i.p. administered CLB-SPD and MGBG was investigated. Polyamine depletion was achieved by administration of 2% DFMO (w/v) in drinking water for 3 days prior to injection of drug. A marked 65-100% increase in MGBG concentration was seen in the small intestine (fig 6.3) with smaller increases in the lung and spleen. Kidney and liver concentrations of MGBG were not affected by DFMO pretreatment. In a separate experiment, mice were innoculated with EATC and the effect of DFMO pretreatment on MGBG uptake investigated. A 2-fold increase in MGBG levels was seen in EATC (fig 6.3).

In contrast, for CLB-SPD it was difficult to see any trends of increased or decreased uptake following DFMO pretreatment since results were quite variable for the different time points (Fig 6.4). Possible small increases in lung, small intestine and ADJ/PC6 tumour were observed at t = 1.5 hr (fig 6.4) whereas for the other tissues a possible trend of decreased levels was seen.

Fig 6.3 The effect of DFMO pretreatment on MGBG levels in tissues and EATC
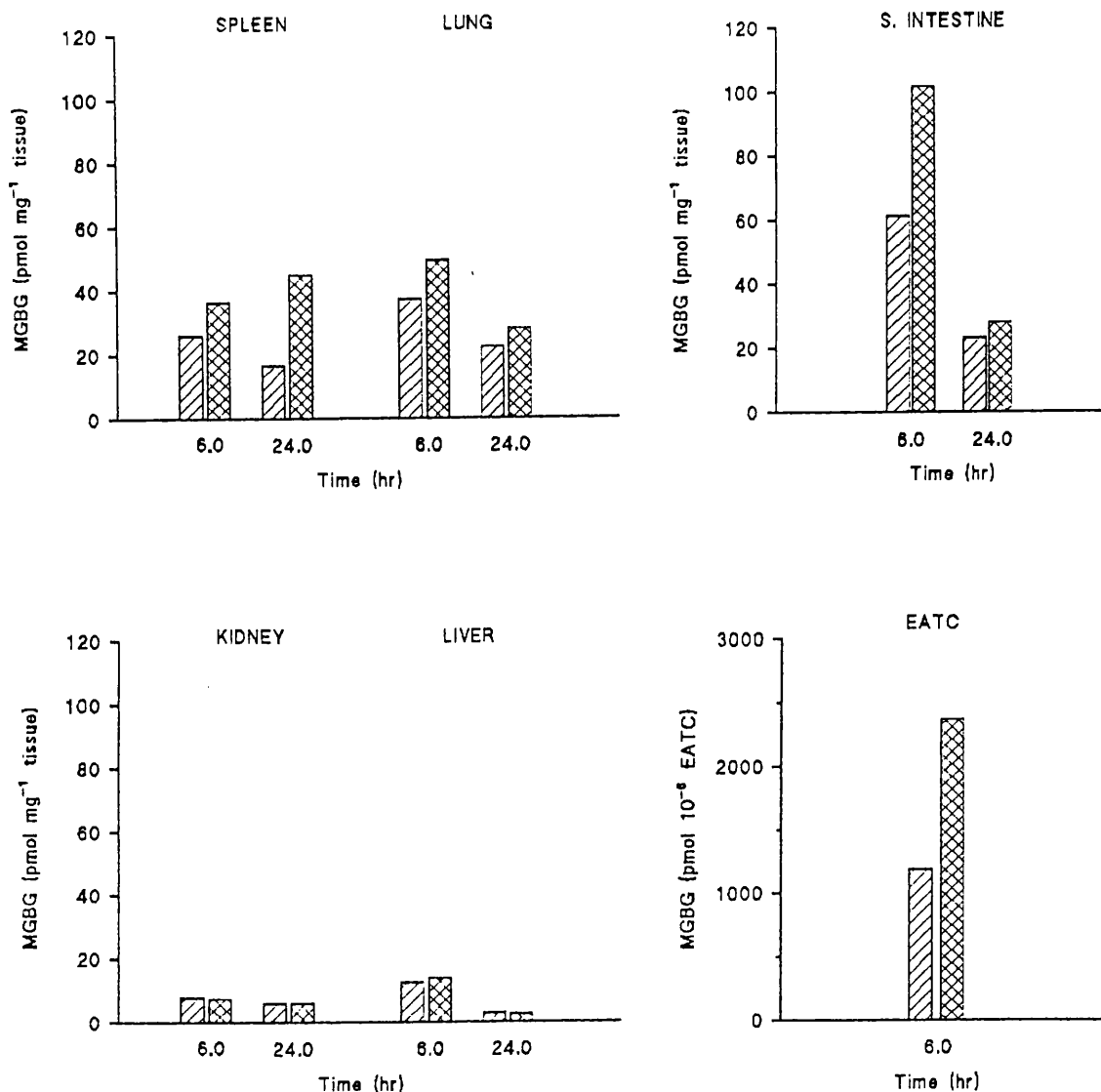
Mice (not EATC innoculated) were administered 2% (w/v) DFMO in drinking water for 72 hr prior to i.p. injection of MGBG (40mg/kg). Tissue levels of MGBG were compared levels in control (no DFMO) animals. In a separate experiment, mice pre-innoculated with EATC were administered 2% DFMO (w/v) in drinking water for 72 hr prior to i.p. injection of MGBG (40mg/kg). MGBG concentrations in EATC were compared to levels in control (no DFMO) mice. Data represent the mean of levels in tissues/EATC from 2 mice per time point and are representative of 2 experiments.

▨ CONTROL   ▨ DFMO pretreated

Fig 6.4   The effect of DFMO pretreatment on CLB-SPD levels in ADJ/PC6 plasmacytoma and other tissues
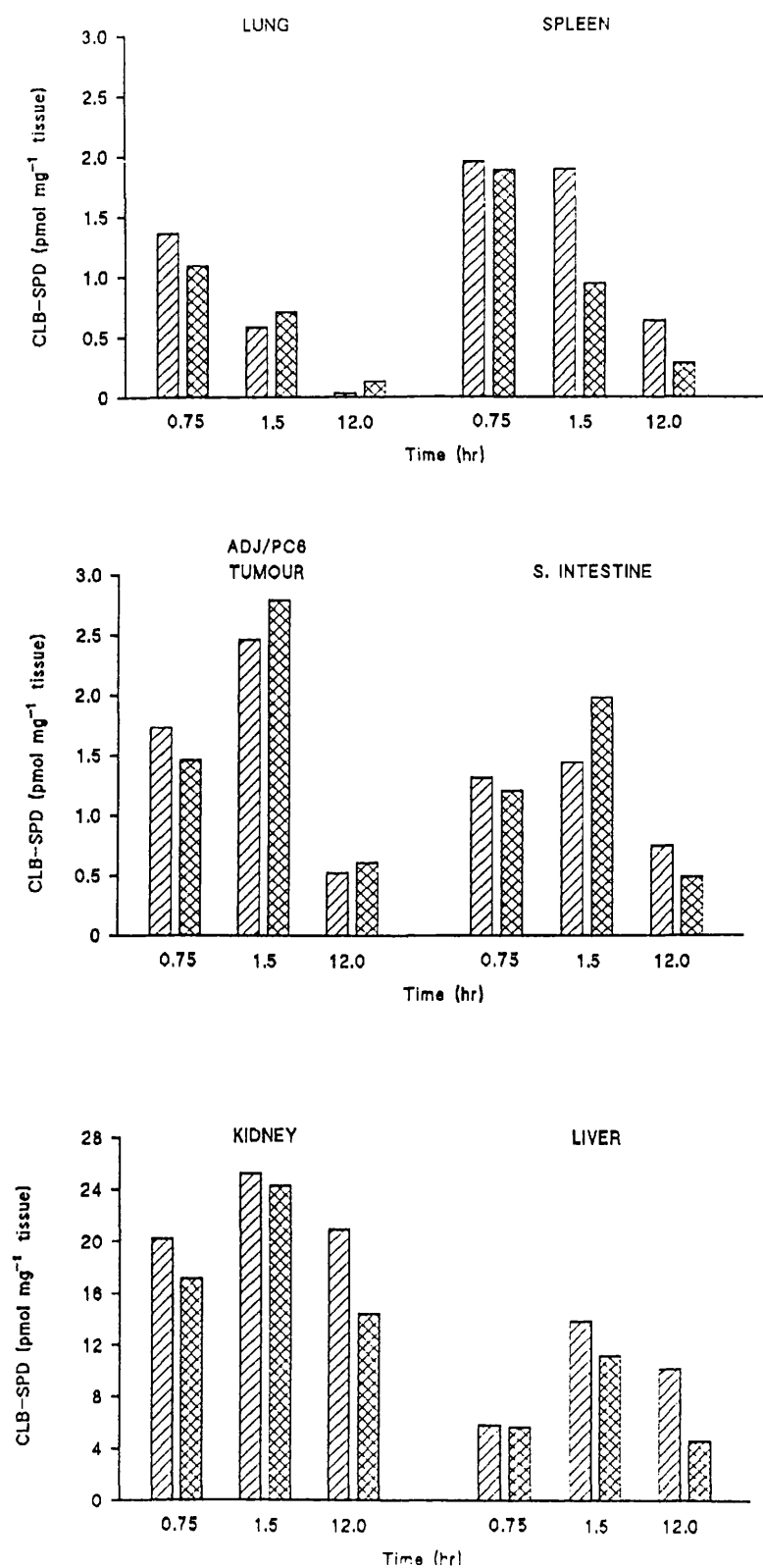
Legend to Fig 6.4 The effect of DFMO pretreatment on
tissue and tumour levels of CLB-SPD conjugate.

   Mice innoculated with ADJ/PC6 plasmacytoma were
administered 2% DFMO (w/v) in drinking water for 72 hr
prior to i.p. injection of CLB-SPD (4mg/kg). Tissue
levels of CLB-SPD were compared to control (no DFMO)
animals by HPLC analysis of extracts of tissue
homogenates. For this study, data represent the mean
levels obtained in tissues from 2 mice.

   ▨ CONTROL   ▨ DFMO pretreated

6.2.3 Effect of continuous, low dose, administration on the tissue distribution of CLB-SPD and MGBG

Attempts were made to alter the tissue distribution of CLB-SPD and MGBG using continuous i.v. infusion (CLB-SPD only) and subcutaneously implanted osmotic minipumps to administer the compounds over longer time periods.

Compared to an equivalent i.p. dose (fig 5.9) a 4 mg/kg infusion of CLB-SPD via a tail vein cannula over a 2 hr time period resulted in a 3 fold increase in ADJ/PC6 tumour, lung, small intestine and splenic levels of CLB-SPD (table 6.1). Liver and kidney levels were unaffected. A 12 hour infusion (4mg/kg) gave a marginal increase in tumour, small intestine, spleen and kidney, but no change in lung concentrations of CLB-SPD compared to i.p.dosing. Liver concentrations were decreased. Statistical analysis (student's t-test) of the tumour data for the 12hr infusion showed that the increase in levels seen compared to i.p. dosing was non-significant.

Using subcutaneously implanted osmotic minipumps, tissue levels of CLB-SPD were in general much lower than following i.p. injection of an equivalent dose, although the highest levels were still found in kidney and liver (Table 6.1). Depletion of polyamines by administration of α-DFMO in the drinking water of mice for 3 days prior to minipump insertion did not result in any increase in tumour tissue levels of CLB-SPD although an increase was seen for lung and small intestine (table 6.1).

The effect of administering MGBG using subcutaneously implanted osmotic minipumps on the tissue distribution of MGBG in EATC bearing mice was also investigated. A linear increase in MGBG concentrations was seen in all tissues, with highest levels being obtained in the small intestine, lung and EATC and the lowest in liver and kidney (fig 6.5).

Table 6.1 Tissue levels of CLB-SPD using continuous infusion and osmotic mini-pumps to administer the drug.

Tissue	CLB-SPD (pmol mg^{-1} tissue)				
	Infusion (i.v.)		Osmotic minipump (s.c.)		bolus (i.p.)
	4mg/kg (2hr)	4mg/kg (12hr)	4mg/kg (24hr) Control	4mg/kg (24hr) DFMO	[a] maximum tissue level
Liver	20.2	9.8	2.9	2.9	22
Kidney	32.9	35.1	7.7	7.7	30
Tumour	7.4	[b] 2.8	0.7	0.8	2.5
Lung	6.6	0.9	n.d	0.25	1.2
S. Intestine	11.1	2.2	n.d	0.8	1.4
Spleen	10.5	5.4	n.d	n.d.	1.9

Mice innoculated with the ADJ/PC6 plasmacytoma were administered CLB-SPD (4mg/kg) either by continuous infusion via a tail vein cannula or from subcutaneously implanted osmotic mini-pumps. 2% DFMO (w/v) was administered in drinking water to one group of mice prior to implantation of the osmotic mini-pumps. Data represent the means of tissues from 2 mice per time point, except [b] where n=3.
Mice were sacrificed immediately following the period of drug delivery. For the continuous infusion experiments, a 5hr infusion of heparinized saline (present in the dead space of the cannula) took place prior to commencement of CLB-SPD delivery.

[a] represents the maximum tissue levels of CLB-SPD obtained following i.p. administration (data taken from fig 5.9)
n.d.= not detectable

Fig 6.5   Tissue levels of MGBG following administration by osmotic mini-pumps.
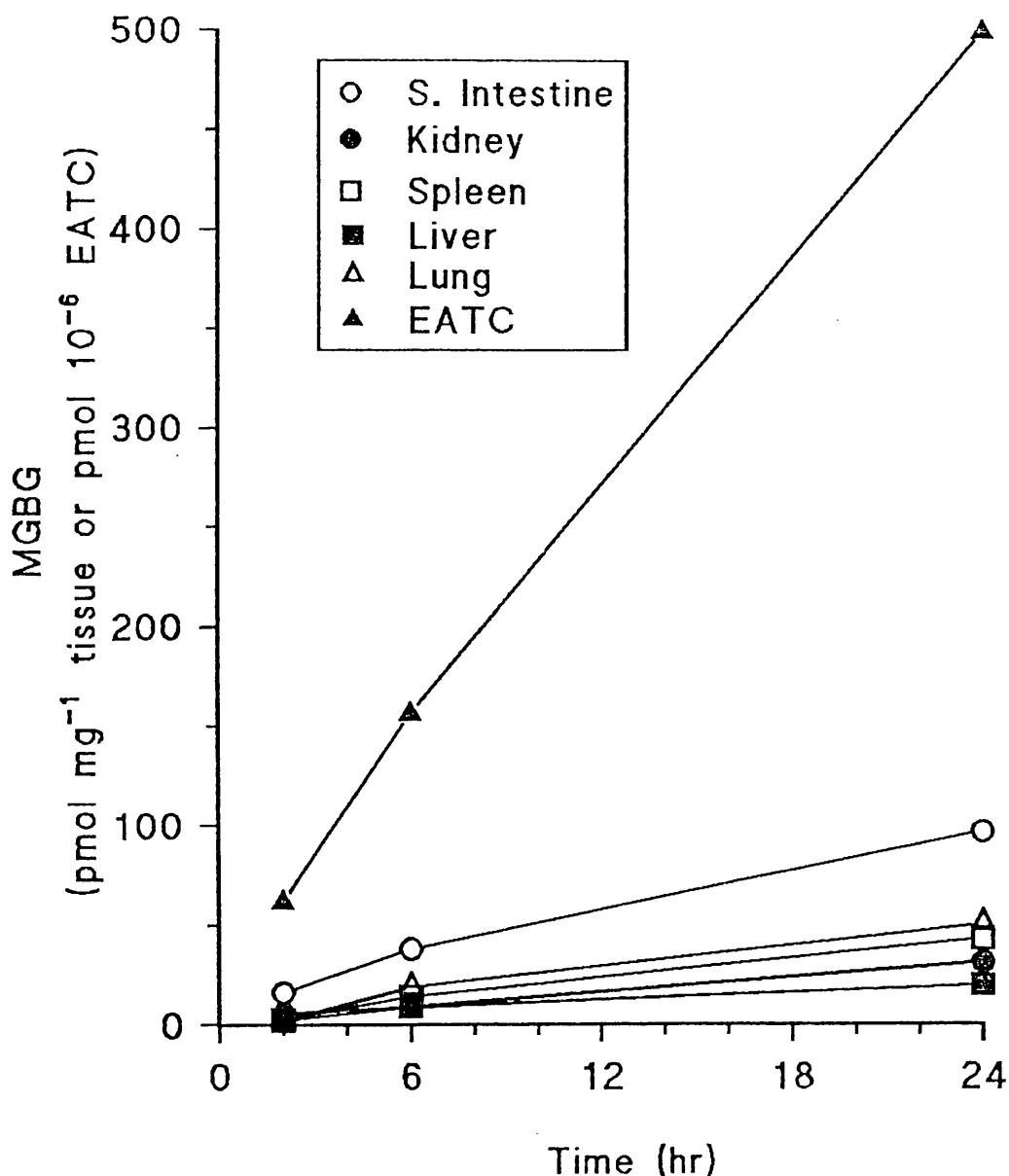
Mice, pre-innoculated with EATC, were administered MGBG from subcutaneously implanted osmotic minipumps (delivery rate = 40mg/kg/24 hr). Mice were sacrificed at the time points shown and PCA extracts of tissue homogenates and EATC analysed by HPLC. Data represent the mean levels of MGBG found in tissues/EATC from two mice per time point.

6.3 Discussion

In chapter 4, CLB-SPD was shown to have a high affinity for the polyamine uptake system of ADJ/PC6 cells *in vitro*. The conjugate also achieved 10-20 -fold higher concentrations than non-conjugated parent compound chlorambucil in ADJ/PC6 cells *in vitro* and *in vivo* (chapter 5). However, a clear role of the polyamine transport system in the uptake of CLB-SPD was difficult to perceive since increased uptake occurred in all tissues studied.

If CLB-SPD utilizes the polyamine uptake system one would have expected some similarities in the tissue distribution of CLB-SPD and the spermidine analogue MGBG. The tissue distribution of CLB-SPD and MGBG in tumour bearing mice was therefore compared. Mice bearing the solid ADJ/PC6 plasmacytoma were used for the CLB-SPD studies. For the MGBG studies, mice innoculated with EATC, were used. Ideally, it would have been better to use the same tumour for both studies, but the MGBG study was carried out prior to obtaining the ADJ/PC6 plasmacytoma. The results obtained demonstrated that the tissue distributions of CLB-SPD and MGBG were, in fact, quite different from each other and suggest that the polyamine uptake system may not play a critical role in the uptake of CLB-SPD.

Following administration of CLB-SPD and MGBG a striking difference in the accumulation of these drugs into the respective tumours was seen. The mean maximal level of CLB-SPD in ADJ/PC6 plasmacytoma was 2.4 pmoles /mg tissue (fig 5.7) whereas for MGBG a maximal level of 325pmoles MGBG/mg EATC was seen (fig 6.3). Expressed as concentrations obtained for equivalent molar doses this represented 7-fold higher levels of MGBG than CLB-SPD being achieved in the respective tumours. High levels of MGBG are also reported to accumulate in L1210 ascites (148). The difference between CLB-SPD and MGBG

accumulation in this study could have been due to the route of administration used and the location of the tumour. Intraperitonal administration would have allowed MGBG to come into direct contact with the EATC which grew as ascites in the peritoneal cavity. This was in contrast to ADJ/PC6 plasmacytoma which is a subcutaneous solid tumour and would have required delivery via the circulatory system. However, the route of administration may not be important since using subcutaneous osmotic minipumps to administer the drugs still gave several fold higher concentrations of MGBG in EATC than CLB-SPD in ADJ/PC6 tumour (fig 6.5 and table 6.1). It would have been useful to measure MGBG uptake in ADJ/PC6 plasmacytoma to obtain a direct comparison, but this tumour was not available when the MGBG distribution studies were performed.

The comparative distribution of CLB-SPD and MGBG in other tissues also gave some unexpected results considering that similar distributions were expected due to their structural analogy. Some striking differences were seen, particularly in their relative distributions in kidney and small intestine (fig 6.1). CLB-SPD accumulated in kidney whereas MGBG accumulated in the small intestine.

The high concentrations of CLB-SPD found in the kidney are in agreement with the high levels of spermidine that accumulate in rat kidney (73) and even higher levels of spermine in mouse kidney (301) following their administration. This accumulation can be explained by the observation that spermine, which is the most basic of the polyamines, and to a lesser extent spermidine, bind to a receptor in the kidney which also binds gentamicin. Gentamycin is reported to bind to mitochondria, microsomal and brush border vesicles and to a lesser extent to macromolecules in the cytosol (304). In contrast, putrescine does not have a high affinity for this receptor and does not accumulate in the kidney (73).

Since CLB-SPD achieved much higher concentrations in kidney than MGBG and it is possible that it may have a similar affinity for the receptor as spermine. This could be because CLB-SPD has four amine groups in its polyamine moiety as a result of the spacer group (fig 3.2). If CLB-SPD had a binding affinity similar to spermine this could explain the much higher concentrations of CLB-SPD that accumulated in kidney compared to MGBG, which being a spermidine analogue had a lower affinity for the receptor. The different binding affinities would also explain why CLB-SPD but not MGBG levels remained high in kidney for 24 hr (fig 6.1). In fact CLB-SPD could still be detected in the kidney 3 days after administration (results not shown).

Since spermine is reported to be nephrotoxic (2), the high concentrations of CLB-SPD seen in the kidney may also cause kidney toxicty. High sustained levels were also seen in the liver (fig 6.1a). The *in vivo* toxicity of CLB-SPD was therefore investigated in a collaborative study (results not shown)(305). Blood urea nitrogen, alanine aminotransferase and aspartate aminotransferase were slightly elevated 24hr after a single dose of CLB-SPD ($24\mu mol/kg$) but had returned to normal 3 days after CLB-SPD administration which suggests that any effects on these tissues were reversible (results not shown) (305). With chlorambucil, blood urea nitrogen was significantly decreased for 3 days following administration. Histological examination demonstrated that chlorambucil showed evidence of systemic toxicty and abnormalities were present in the liver, spleen, lungs, adrenals, pancreas and intestine 5 days after administration. There was also evidence that apoptosis had occurred in the intestine and thymus. With CLB-SPD, there was evidence of spleen and thymus atrophy, iron deposits in the spleen and an increase in the number of apoptotic epithelial cells in the intestine. However, the effects seen with CLB-SPD were less severe than those seen for

chlorambucil. The toxicity of MGBG was also investigated and there was evidence of renal damage. Therefore despite its accumulation and retention in the kidney, CLB-SPD demonstrated less toxicity than MGBG in this tissue.

Another striking difference in the tissue distribution of CLB-SPD and MGBG was the accumulation of MGBG but not CLB-SPD in the small-intestine. Similar observations of high MGBG accumulation in the small intestine are reported in the literature (73,74,181). In addition, the administration of spermidine and putrescine to rats also results in the accumulation of these compounds in the small-intestine (73). Therefore CLB-SPD appeared to have a lower affinity for intestinal tissue than the other polyamines. These results indicate that the tissue levels and distributions of CLB-SPD differ from MGBG and the other polyamines.

The potential toxicity of polyamine analogues to tissues possessing the polyamine uptake system has already been discussed (chapter 1). The accumulation of MGBG in the small intestine in this and other studies (73,74,181) is probably responsible for its reported toxicity to this tissue (182,187). The poorer CLB-SPD accumulation in the small-intestine may be beneficial in that it may be less toxic. In addition, the poor accumulation of CLB-SPD in the lung suggests that the anticipated CLB-SPD toxicity to this tissue, which was expected due to the presence of a polyamine uptake system in the lung (129,167), may not occur after all. Heston et al. (73) and Kallio et al. (74) did not measure polyamine uptake in the lung so no comparison between this and their studies could be made. A study by Herr et al., however, showed that accumulation of MGBG was greater in the lung than tumour tissue (303) (table 6.2).

The observation that CLB-SPD was not accumulated to any great extent in ADJ/PC6 tumour suggests that conjugation of chlorambucil to a polyamine carrier has

not targeted tumour tissue as well as anticipated. This could be due to poor activity of the polyamine uptake system in ADJ/PC6 tumour cells *in vivo*, or possibly the inability of CLB-SPD to utilize the polyamine uptake system. In view of these results and those from chapter 5, it appears that the polyamine uptake system does not play a critical role in the delivery of CLB-SPD.

This suggestion is supported by the observation that DFMO pretreatment had little effect on CLB-SPD uptake in any tissue, whereas increases in both MGBG and polyamine uptake occur in at least one tissue (in particular small intestine and tumour tissue) following DFMO pretreatment (table 6.2). One would have expected that polyamine depletion would result in a selective increase in the uptake of polyamines and polyamine analogues by rapidly proliferating tissues possessing the polyamine uptake system, eg; small intestine and tumour, as demonstrated in a number of studies (table 6.2) (73,74,146,148,188,303).

The effect of DFMO on MGBG and CLB-SPD accumulation was quite different. Only a minimal increase in CLB-SPD uptake into ADJ/PC6 plasmacytoma was seen (fig 6.4) in DFMO pretreated mice, compared to MGBG where uptake by EATC was increased 2-fold (fig 6.3). DFMO is also reported to increase the uptake of MGBG in L1210 cells (188), renal adenocarcinoma (303) and Dunning R-3327 rat prostate derived tumour (306) *in vivo*. A selective increase in the accumulation of putrescine into EMT6 tumour following DFMO pretreatment is also reported (146).

MGBG, but not CLB-SPD, uptake was substantially increased in the small intestine by DFMO pretreatment (fig 6.3 and 6.4). Increased MGBG accumulation is also reported by Kallio *et al.* (74) in non-tumour bearing mice (table 6.2). An interesting observation is the observed increase in MGBG uptake into the small intestine of normal but not L1210 bearing mice pretreated with DFMO

Table 6.2 Comparison of the effect of DFMO on tissue and tumour levels of MGBG and CLB-SPD

Reference	This study	This study	Heston et al. (73)	Kallio et al. (74)	Kramer et al. (188)	Kramer et al. (188)	Herr et al. (303)
Compound	CLB-SPD	MGBG	MGBG	MGBG	MGBG	MGBG	MGBG
Route of administration	(i.p.)	(i.p.)	(i.v.)	(i.p.)	(i.v.)	(i.v.)	(i.p.)
Tumour used	ADJ/PC6 (mouse)	EATC (mouse)	Prostate (rat)	No tumour (mouse)	L1210 (mouse)	No tumour (mouse)	renal adenocarcinoma (mouse)
Tissue				DFMO EFFECT			
Tumour	± (0.8)	++ (27)	no effect (3.0)		++ (14.3)		++ (0.2)
small intestine	± (0.11)	++ (1.6)	no effect (0.7)	++ (5.0)	++ (2.9)	+	no effect (1.2)
lung	± (0.04)	+ (0.45)	ND	ND	ND	ND	no effect (0.5)
spleen	- (0.13)	+ (0.45)	ND	+ (1.5)	++ (1.4)	ND	no effect (0.38)
kidney	- (1.8)	no effect (0.6)	++ (0.36)	no effect (1.0)	++ (2.3)	ND	no effect (0.84)
bone marrow	ND	ND	ND	++ (3.5)	ND	ND	ND
liver	- (1.0)	no effect (1.0)	-- (1.0)	no effect (1.0)	++ (1.0)	ND	- (1.0)

ND = not determined

Values in parentheses are tissue levels of drug in control (i.e., no DFMO) animals, relative to liver concentrations.

Key to DFMO effect:

++ = > 40% increase; + = 25 - 40% increase; ± = variable effect; - = 25 - 40% decrease; -- = > 40% decrease

(188) (table 6.2), suggesting competition for MGBG uptake by tumour cells. A similar observation was made in this study, where in EATC innoculated mice, DFMO was less effective at increasing the levels of MGBG in the small intestine (results not shown). Putrescine uptake by the small intestine is reported to be both increased (73) and decreased (146) by DFMO in tumour bearing rodents whereas spermidine uptake is not affected (73). Comparison of the results obtained for CLB-SPD in this study to those in the literature for other polyamines and polyamine analogues suggests that CLB-SPD is not acting as a typical polyamine with regards to its uptake characteristics.

In order for DFMO pretreatment to increase the uptake of polyamines into tissues with a polyamine uptake system, polyamine depletion must be achieved. It is possible that polyamine depletion was not achieved in the ADJ/PC6 plasmacytoma. In general it is more difficult to deplete polyamines *in vivo* than *in vitro* since *in vivo,* polyamines can be replenished from exogenous sources (2,156,157). In addition, with α-DFMO, it is more difficult to deplete spermidine than putrescine in tissues and tumours (74,188,307). This is especially apparent in slow growing tumours (188) and tissues (148). The observation that increased MGBG accumulation occurs in L1210 cells *in vivo* only if spermidine is depleted (72,147) is consistent with the suggestion that DFMO induced spermidine depletion may not have been sufficient to stimulate increased accumulation of CLB-SPD in the ADJ/PC6 tumour (this study), or MGBG and spermidine accumulation in other solid tumours (73). In addition, spermine levels are not depleted by DFMO and if CLB-SPD is acting like spermine, this could also offer an explanation as to why no increase in uptake was seen.

A further possible reason for the lack of increased CLB-SPD accumulation with DFMO in ADJ/PC6 tumour is that some tumours are over-producers of ODC

(308,309) and it is difficult to deplete their polyamine levels. In ADJ/PC6 cells *in vitro*, DFMO pretreatment gave only a 2-fold increase in spermidine uptake compared to EATC where a 6-fold increase was seen (chapters 3 and 4). Therefore, it may be difficult to deplete spermidine in ADJ/PC6 cells *in vitro* and *in vivo*. Alternatively the ADJ/PC6 cells may not have the ability to increase their rate of polyamine uptake to such an extent as EATC following polyamine depletion.

Attempts to increase tumour selectivity of CLB-SPD by utilization of pharmacokinetics were made. In these studies, CLB-SPD was administered by continuous i.v. infusion and osmotic minipumps. The rationale behind this was that tumour selectivity should be enhanced by maintaining plasma levels of CLB-SPD near the Km for uptake of the compound (267). It was believed that administration by i.v. infusion or osmotic minipumps would meet these requirements better than a bolus dose.

A 2 hour infusion of CLB-SPD (4mg/kg) resulted in a 3-fold increase in tumour tissue levels (table 6.1) although this increase was not tumour selective since similar, if not greater increases were seen in lung, small intestine and spleen compared to an i.p. dose (fig 5.9 and table 6.1). However, since kidney and liver levels were not affected this suggests that some degree of selectivity to tissues with a polyamine uptake system may have been achieved. Infusion over a longer time period (12 hr) resulted in a minimal, statistically non-significant, increase in tumour levels of CLB-SPD compared to i.p. dosing (table 6.1). In a further experiment (results not shown) a 12hr infusion of 1 mg/kg CLB-SPD resulted in tumour levels approximately one quarter those achieved with a 4 mg/kg 12hr infusion. These results suggest that manipulating doses and pharmacokinetics did not alter the uptake of the conjugate specifically into tumour tissue and that, with this polyamine conjugate at least, selectivity to tumour

194

tissue was not achieved.

The administration of CLB-SPD (4mg/kg) by
subcutaneously implanted osmotic minipumps over a 24hr
period, resulted in a reduction in tissue levels compared
to an i.p. dose or continuous infusion (table 6.1). This
may have been due conjugate becoming bound subcutaneously
following release from the minipump. In contrast, MGBG
tissue levels were of the same order of magnitude
following i.p. or osmotic minipump administration (fig
6.2 and 6.5) which suggests that such binding did not
occur for MGBG. Also, with MGBG, some selectivity to
EATC, small intestine and lung tissue was apparent (fig
6.5) since the highest levels were achieved in these
tissues. Therefore it would appear that continuous low
dose delivery of MGBG may increase selectivity to tissues
with the polyamine uptake system relative to other
tissues with a less active uptake system.

Using osmotic minipumps, some tumour selectivity
of CLB-SPD compared to lung and small intestine was
achieved since CLB-SPD levels were below the minimum
level of detection in these tissues (table 6.1).
However, prior administration of DFMO to mice gave
enhanced accumulation in the lung and small intestine but
not ADJ/PC6 tumour (table 6.1). This confirms the earlier
observation that it may be difficult to deplete
polyamines in the ADJ/PC6 plasmacytoma or else that the
polyamine uptake system does not play an important role
in the delivery of CLB-SPD.

In summary, the comparison of the tissue
distribution of CLB-SPD, MGBG, spermidine and putrescine
in this and previous studies demonstrates that there are
quite striking differences in their uptake into tissues.
However, MGBG appeared to have greater similarity to the
natural polyamines in its uptake than CLB-SPD, especially
with respect to DFMO effects. The distribution of CLB-
SPD, in particular its high affinity for the kidney,
appeared to be more like spermine, but since very few

reports on the distribution of exogenous spermine have been made it was difficult to compare this phenomenon. Since tumour tissue levels of CLB-SPD after i.p. administration were relatively low and tumour levels were not enhanced by utilization of pharmacokinetics or polyamine depletion with DFMO, this would suggest that the polyamine uptake system does not play a critical role in the uptake of CLB-SPD into tissues.

CHAPTER 7 - The relationship between polyamine uptake and the *in vitro* toxicity of CLB-SPD to four human ovarian carcinoma cell lines

7.1 Introduction

The results from chapters 5 and 6 suggest that the increased tissue delivery of CLB-SPD compared to chlorambucil occurs in all tissues and is not specific for tumours or other tissues with an active polyamine uptake system. The enhanced tissue levels obtained with CLB-SPD compared to chlorambucil may have been due to high intracellular binding of the conjugate which did not occur with chlorambucil. CLB-SPD did not follow a tissue distribution pattern typical of polyamines and its uptake was not increased in any tissue by DFMO pretreatment. The role of the polyamine uptake system for the uptake of CLB-SPD and its influence on the toxicity of CLB-SPD was not clear.

In order to investigate the influence of polyamine uptake on the toxicity of the conjugate, four human ovarian carcinoma cell lines (PXN/94, SKOV-3, HX/62 and 41M) were studied. These cell lines have been developed for possible use in a panel of human tumour cell lines for *in vitro* screening of antitumour agents to replace *in vivo* animal models (271). They have differing sensitivities to platinum drugs and melphalan, with SKOV-3 and HX/62 cells tending to be resistant, 41M cells sensitive and PXN/94 cells having a variable response depending on the drug used (231). The *in vitro* toxicity of CLB-SPD and chlorambucil to each cell line was investigated. The kinetics of spermidine uptake in the different cell lines, together with the ability of CLB-SPD to inhibit uptake, were also studied. A comparison was made between the kinetic parameters for each cell type and the *in vitro* sensitivity to CLB-SPD, to determine if any relationship existed between polyamine

uptake, the affinity of CLB-SPD for the uptake system and its *in vitro* toxicity. In addition, the uptake of CLB-SPD into PXN/94 cells (CLB-SPD sensitive) and SKOV-3 cells (CLB-SPD resistant) was measured using HPLC to determine whether differences in intracellular concentrations of CLB-SPD were responsible for the different sensitivities of the cell lines.

7.1 Results

7.2.1 Comparison of the *in vitro* toxicity of CLB-SPD and chlorambucil to PXN/94, SKOV-3, HX/62 and 41M cell lines.

The *in vitro* toxicity of CLB-SPD and chlorambucil to PXN/94, SKOV-3, HX/62 and 41M cell lines, following a 1 hr exposure, was investigated and IC_{50} values compared (Table 7.1). The SKOV-3 cells were the least sensitive to chlorambucil ($IC_{50}=142\mu M$) and 41M cells most sensitive ($IC_{50}=24\mu M$). In all the cells, CLB-SPD was more toxic than chlorambucil, with a striking 200-fold enhanced activity in PXN/94 cells. The differential between CLB-SPD and chlorambucil toxicity was less in the other cell lines (Table 7.1).

7.2.2 Characterisation of spermidine uptake in PXN/94, SKOV-3, HX/62 and 41M cell lines.

The kinetics of spermidine uptake in the four human ovarian carcinoma cell lines were investigated. All four cell lines had concentration dependent, saturable uptake systems for spermidine. The Km and Vmax values obtained are shown in table 7.2. Little variation was seen in the Km values for the different cell lines which ranged from $0.36\mu M$ (HX/62) to $0.59\mu M$ (PXN/94).

198

Table 7.1   *In vitro* toxicity of CLB-SPD conjugate and chlorambucil to four human ovarian carcinoma cell lines.

CELL LINE	IC_{50} (μM) (CLB-SPD)	IC_{50} (μM) (CHLORAMBUCIL)	FOLD DIFFERENCE
PXN/94	0.42 ± 0.02	80.7 ± 0.67	192
SKOV-3	16.5 ± 1.0	142 ± 9.87	8.6
HX/62	25.6 ± 5.8	73.0 ± 4.04	2.8
41 M	4.1 ± 0.67	23.7 ± 2.8	5.7

PXN/94, SKOV-3, HX/62 and 41M human ovarian carcinoma cells were incubated with CLB-SPD (0-160μM) and chlorambucil (0-160μM) for 1 hr and fresh medium added for a further 71 hr. ^{3}H-thymidine was added for the final 2 hr of the incubation except with HX/62 cells where a 4 hr pulse was used. The concentration of each drug giving a 50% inhibition of ^{3}H-thymidine incorporation into DNA compared to control cells (IC_{50}) was calculated. Data represent the mean ± s.e.m. of at least 3 experiments.

Table 7.2 Kinetics of spermidine uptake in four human ovarian carcinoma cell lines and its inhibition by CLB-SPD.

CELL LINE	Km (μM)	*Vmax	Ki (μM)$_{(CLB-SPD)}$
PXN/94	0.59 ± 0.07	0.60 ± 0.13	10.1 ±1.9
SKOV-3	0.37 ± 0.04	2.63 ± 0.31	4.56 ± 1.4
HX/62	0.36 (2)	2.97 (2)	3.45 (2)
41 M	0.41 ± 0.06	1.39 ± 0.10	5.42 ± 0.82

* pmoles min^{-1} 10^{-5} cells

Human ovarian carcinoma cells (1×10^5) were incubated with ^{14}C-spermidine (0.125-5μM) alone or in the presence of CLB-SPD (2 or 10μM) for 30 min. Km, Vmax and Ki values were calculated by analysis of reciprocal rate plots. Data represent the mean ± s.e.m. of at least 3 experiments performed in duplicate except for HX/62 cells where n=2.

Larger differences were seen in the maximum velocities (Vmax) which ranged from 0.6pmoles/min/10^{5}cells(PXN/94) to 3 pmoles/min/10^{5}cells (HX/62). The temperature dependence of spermidine uptake was investigated in PXN/94 and SKOV-3 cells and found to be reduced at 4°C compared to 37°C in both cell lines (Fig 7.1).

7.2.3 Inhibition of spermidine uptake by CLB-SPD in PXN/94, SKOV-3, HX/62 and 41M cell lines.

The ability of CLB-SPD to inhibit radiolabelled spermidine uptake into the human ovarian carcinoma cells was investigated. In all cell lines, spermidine uptake was inhibited competitively by CLB-SPD. The Ki values obtained are shown in table 7.2. CLB-SPD was most effective at inhibiting spermidine uptake in HX/62 cells (Ki = 3.5μM) and least effective in PXN/94 cells (Ki = 10.1μM).

7.2.4 *In vitro* uptake of CLB-SPD in PXN/94 and SKOV-3 cells.

The *in vitro* uptake of CLB-SPD in PXN/94 and SKOV-3 cells was compared using HPLC to measure levels. For both cell lines uptake was greater at 37°C than 4°C (Fig 7.2) and slightly higher levels of CLB-SPD were achieved in SKOV-3 than PXN/94 cells. Subtracting the uptake at 4°C from that at 37°C allowed the kinetics of the temperature dependent contribution to CLB-SPD uptake to be estimated. For both cell lines the rate of CLB-SPD uptake increased with concentration and was saturable (Fig 7.3a). Km and Vmax values were calculated from Lineweaver-Burk plots (Fig 7.3b) and were as follows:

PXN/94 ; Km = 6.9μM, Vmax = 1.1 pmoles/min/10^{6}cells
SKOV-3 ; Km = 2.9μM, Vmax = 1.7 pmoles/min/10^{6}cells

Fig 7.1 The effects of temperature on spermidine uptake in PXN/94 (a) and SKOV-3 (b) cells.

A

B
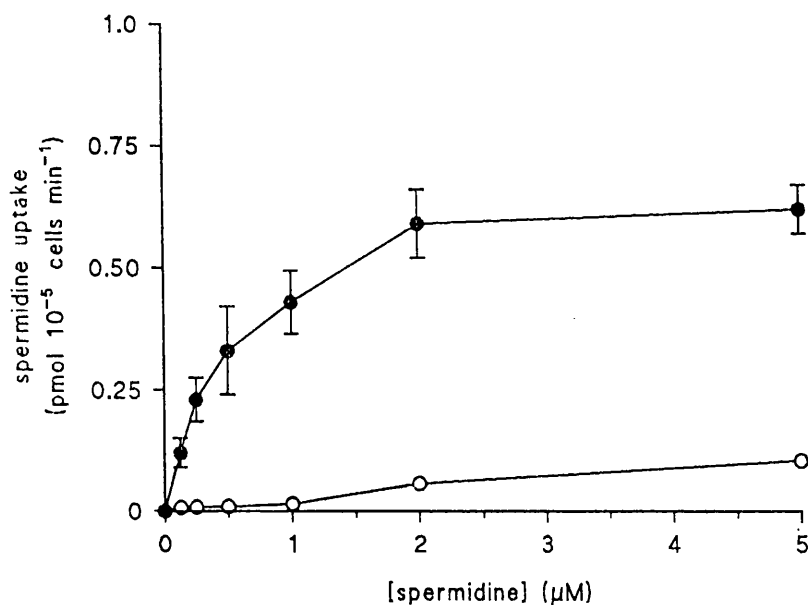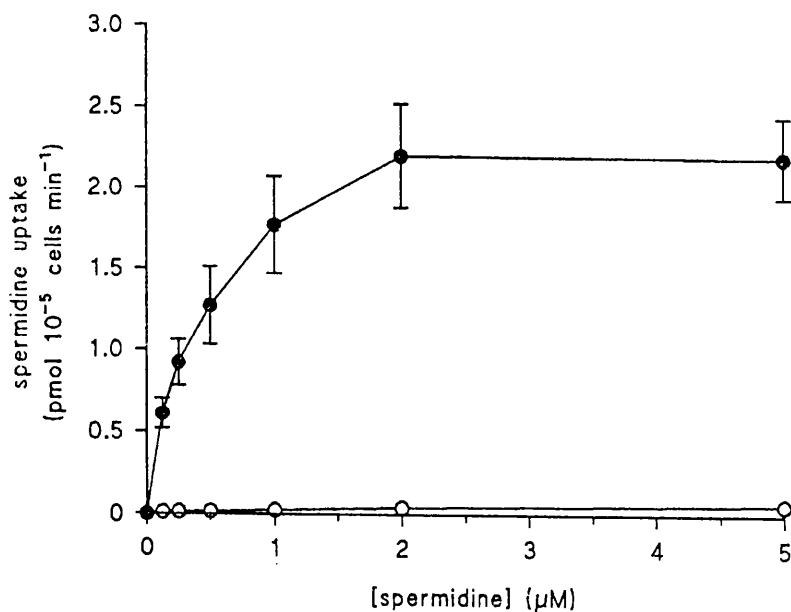
1x10^5 PXN/94 (a) or SKOV-3 (b) cells were incubated in duplicate for 30 min with ^{14}C-spermidine (0.125 - 5.0 μM) at 37°C (●) and 4°C (○) and the uptake of ^{14}C-label measured. Data represent the means ± s.e.m. of 4 experiments at 37°C and the means of two experiments at 4°C.

Fig 7.2 *In vitro* **uptake of CLB-SPD conjugate in PXN/94 and SKOV-3 cells at 37°C and 4°C.**
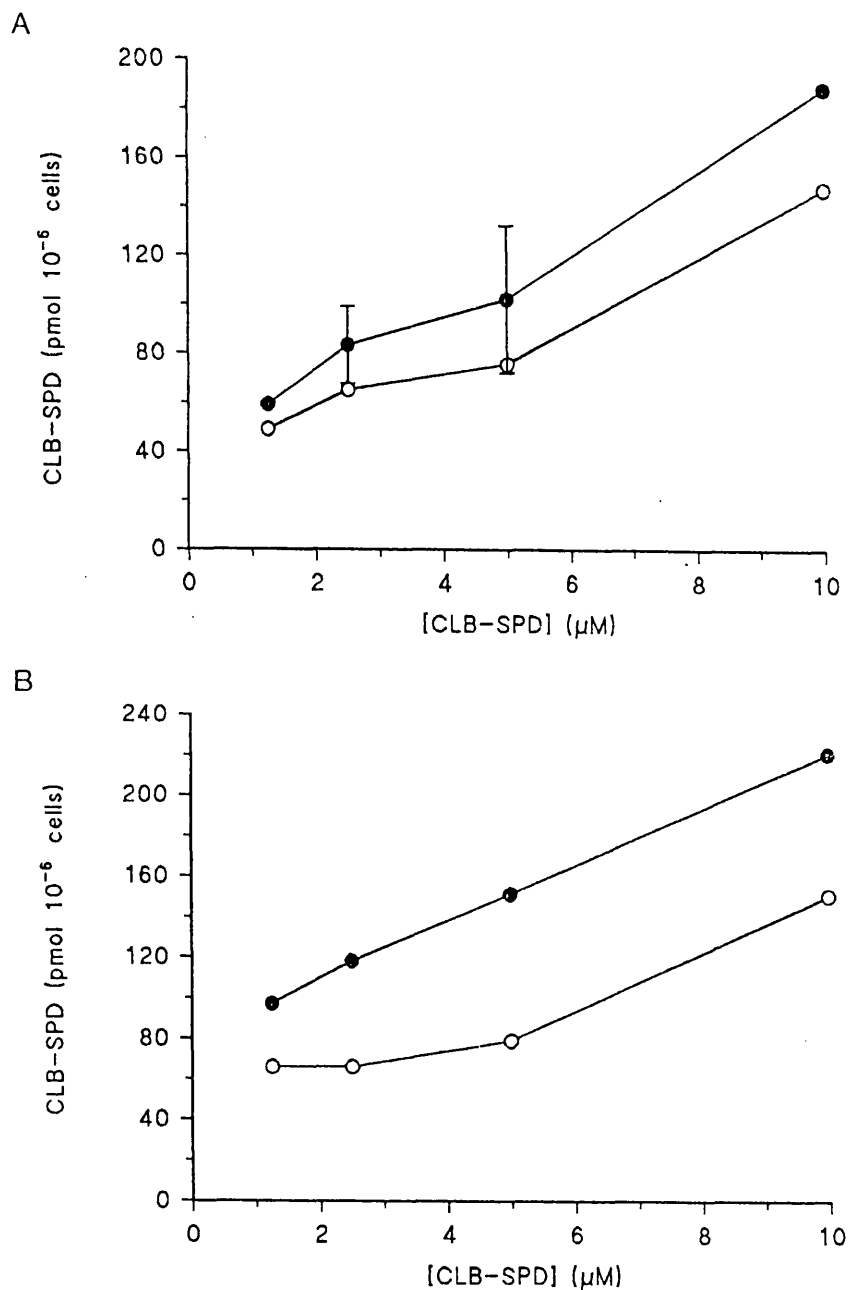
Monolayers of PXN/94 (a) and SKOV-3 cells (b) (3×10^6) were incubated with CLB-SPD conjugate (1.25 - 10μM) for 60 min at 37°C (●) and 4°C (O). Cell extracts were analysed by HPLC to determine CLB-SPD levels. Data represent the means ± S.D. of at least 3 experiments except where error bars are not shown and n=2.

Fig 7.3 Kinetics of temperature dependent *In vitro* uptake of CLB-SPD in PXN/94 and SKOV-3 cells.

A

B
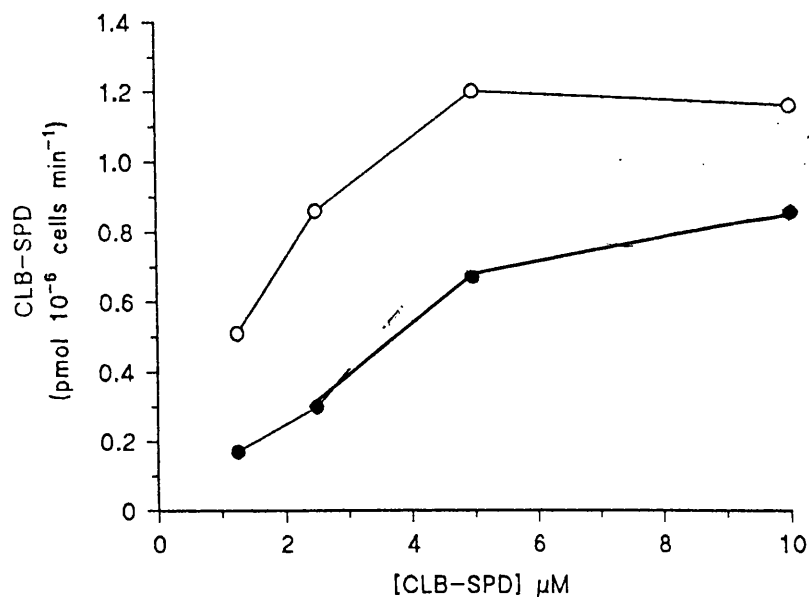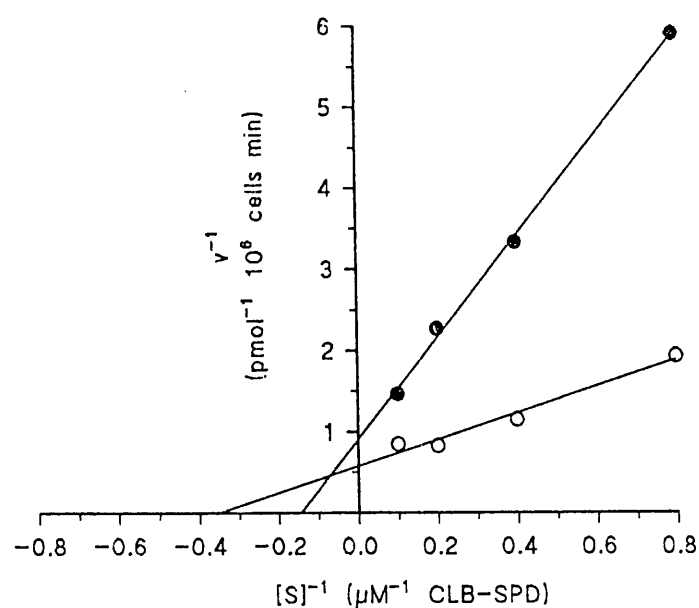
PXN/94 (●) or SKOV-3 (O) cells (3x10^{6}) were incubated for 60 min with CLB-SPD (1.25-10μM) at 37°C and 4°C. Cell extracts were analysed by HPLC. CLB-SPD uptake occuring at 4°C was subtracted from that for 37°C to obtain linear (a) and reciprocal (Lineweaver-Burk) (b) rate plots from which Km and Vmax values were obtained.

7.3 Discussion

Previous studies (chapters 5 and 6) indicated that increased uptake of CLB-SPD compared to chlorambucil may not have been due to the conjugate utilizing the polyamine uptake system. It was possible that the increased accumulation seen was due to intracellular binding of CLB-SPD. In this study, the *in vitro* toxicity of CLB-SPD and chlorambucil to four human carcinoma cell lines (PXN/94, SKOV-3, HX/62 and 41M) was compared and the kinetic parameters for spermidine uptake and the affinity of CLB-SPD for the uptake system. The results were compared to help elucidate whether polyamine uptake and/or the affinity of the CLB-SPD conjugate for the uptake system were related to the toxicity of the conjugate.

All four cell lines demonstrated saturable and concentration dependent spermidine uptake. In those cells tested (PXN/94 and SKOV-3) spermidine uptake was also temperature dependent (fig 7.1). A 5-fold difference in the maximal velocity of spermidine uptake was observed with PXN/94 cells having the lowest Vmax, and HX-62 cells the highest (table 7.2). Less variation in the Km values was seen.

The human ovarian carcinoma cells demonstrated varying degrees of sensitivity to CLB-SPD with PXN/94 cells being the most sensitive ($IC_{50}=0.4\mu$M) and SKOV-3 and HX/62 cells the most resistant ($IC_{50}=$ 16.5 and 25.6μM respectively)(table 7.1). In all four cell lines CLB-SPD was more toxic than chlorambucil following a 1 hr exposure. The most striking observation was the 200-fold increased sensitivity of PXN/94 cells to CLB-SPD compared to chlorambucil. Even in SKOV-3 cells, which were very resistant to chlorambucil, there was an 8-fold increase in toxicity of the conjugate compared to chlorambucil (table 7.1). These results provide further evidence in support of the hypothesis that conjugation of

chlorambucil to a spermidine carrier increases its
toxicity to tumour cells possessing the polyamine uptake
system.

Comparison of the IC_{50} values for CLB-SPD with the
Km and Vmax for spermidine uptake and the Ki for CLB-SPD
in the different cell lines, however, suggests that these
parameters were not directly related to their sensitivity
to CLB-SPD. PXN/94 cells, which were the most sensitive
to CLB-SPD, had the lowest Vmax and highest Km for
spermidine uptake. In contrast, HX/62 cells, which were
the most resistant to CLB-SPD had the highest Vmax and
lowest Km. This suggests that spermidine had a slightly
higher affinity and more rapid rate of uptake in the less
sensitive cell line. In addition the affinity of CLB-SPD
for the uptake system, as assessed by Ki values, was
lowest for PXN/94 cells (sensitive) and highest for HX/62
cells (resistant). The data for all four cell lines
suggests that their sensitivity was actually inversely
proportional to the affinity of spermidine and CLB-SPD
for the uptake system. This was in total contrast to the
original hypothesis that the higher the affinity of the
polyamine-cytotoxic conjugate for the uptake system, as
judged by its Ki value, the greater the expected
toxicity.

The sensitivity of the four cell lines to CLB-SPD
and chlorambucil was, however, in agreement with the
results obtained for these cells with platinum drugs and
melphalan by Mistry et al who report that SKOV-3 and
HX/62 cells demonstrate a degree of resistance, 41M cells
are sensitive and that PXN/94 cells demonstrate
differential toxicity depending on the drug used (231).
In this study, PXN/94 cells were fairly resistant to
chlorambucil but very sensitive to CLB-SPD.

Further studies on the uptake of CLB-SPD into a
sensitive and resistant cell line were carried out to
investigate whether there were, perhaps, any differences
in the cellular levels of CLB-SPD achieved in sensitive

and resistant cell lines. For this study, PXN/94 (sensitive) and SKOV-3 (resistant) cells were used. HX/62 cells (the most resistant) were not used because they had a long doubling time and it was time consuming to grow up the large numbers required. CLB-SPD uptake was temperature dependent in both cell lines. The differential in uptake between 4°C and 37°C (Fig 7.2) was not very large, unlike that seen for spermidine uptake in these cells (fig 7.1). This could be due to high extracellular binding of the conjugate to the cells or the plastic culture vessel used. Binding of CLB-SPD to the plastic culture vessel was observed when flasks were incubated with the drug in the absence of cells (results not shown). An alternative explanation for the small differential in uptake between 37°C and 4°C is that there could be a high diffusional element of uptake with only a small contribution from active uptake occurring. Further analysis of the data, however, suggests that the diffusional element may not be as high as it first appears. Exposure of PXN-94 cells to $10\mu M$ CLB-SPD at 4°C resulted in an apparent concentration of 140pmoles CLB-SPD/10^6 cells (fig 7.2a). If, for example, the intracellular volume of 10^6 cells was approximately $2\mu l$, this means that the intracellular concentration of CLB-SPD was $70\mu M$, which is 7-fold greater than the extracellular concentration. It seems unlikely that diffusion alone could account for this apparent high intracellular/ extracellular ratio, especially since intracellular binding, which could possibly result in an high ratio, would be lower at 4°C. It seems likely that non-specific binding to culture vessels (which are coated to help cells adhere) may be responsible for the apparent high levels obtained at 4°C.

The levels of CLB-SPD were slightly higher in SKOV-3 than PXN/94 cells both before and after correcting for non-temperature dependent uptake (fig 7.2 and fig 7.3). The kinetics for the temperature dependent element

of CLB-SPD uptake were calculated and compared (fig 7.3).
The affinity of CLB-SPD for PXN-94 cells (Km=6.9μM) was
less than SKOV-3 cells (Km=2.9μM) which was in agreement
with affinity of CLB-SPD (as judged by Ki values) for the
uptake system in the two cell lines (table 7.2). The
Vmax values for CLB-SPD uptake also followed the same
trend as spermidine uptake for both cell lines, although
the maximum rate of CLB-SPD uptake was approximately 5
(PXN/94) and 15 (SKOV-3) -fold lower than spermidine
uptake. Comparison of Km values indicated that
spermidine had an approximate 10-fold higher affinity for
the uptake system than CLB-SPD in both cell lines. A
similar observation was made in ADJ/PC6 cells in chapter
5.

These results suggest that CLB-SPD is transported
by the polyamine uptake system to some extent, but that
the affinity for, and transport by, the polyamine uptake
system is not particularly great. It is difficult to
draw conclusions regarding the effect of the affinity of
CLB-SPD for the uptake system on the toxicity of CLB-SPD,
since it was not clear exactly how large the diffusional
element of uptake was. The results do highlight the fact
that despite apparent similar intracellular levels being
achieved, large differences in the sensitivity of PXN-94
and SKOV-3 cells to CLB-SPD were observed. It is
therefore likely that alternative mechanisms were
involved in their differential sensitivity

In agreement with Hills et al. (271), there
appeared to be no apparent relationship with cell
doubling times of PXN/94 and SKOV-3 cells and their
sensitivity (results not shown). However the resistant
HX/62 cell line had a slower doubling time which could
explain the resistance to CLB-SPD in this cell line.
The role of GSH and GST in the resistance of
tumour cells to alkylating agents, including
chlorambucil, has been widely reported (230,231,

232,233,234,235,236). Mistry *et al.* also investigated the role of GSH and total GST activity in the toxicity of melphalan and platinum drugs to the human ovarian carcinoma cells used in this study (231). Low GSH levels, as found in 41M cells, conferred sensitivity and an interesting observation in this study was the sensitivity of 41M cells to CLB-SPD. However, normal GSH levels were reported for PXN/94 and SKOV-3 cells and so differences in GSH were probably not responsible for the differential sensitivity to CLB-SPD seen in this study. The role of GSH in protecting against CLB-SPD toxicity could be investigated by depleting intracellular GSH with BSO (buthionine sulfoximine).

GST enzymes catalyse the conjugation of alkylating agents to GSH and their subsequent inactivation. Low enzyme activity should render the cells more sensitive to alkylating agents. Enzyme levels are reported to be similar in PXN-94 and SKOV-3 cells (231) and therefore no correlation between sensitivity to CLB-SPD and total GST activity was apparent. In fact, HX/62 cells which have low GST activity, were actually resistant, rather than sensitive, to CLB-SPD. The results of this study are in agreement with Mistry *et al* who also found no correlation between total GST levels and IC_{50} values with the platinum drugs (231). However, since individual isoenzymes were not measured, in particular the α-isoenzyme which is thought to most important for catalysing the conjugation reaction (225,226), no definite conclusions can be made.

An alternative mechanism for alkylating agent resistance is altered formation and removal of DNA cross links (229). However, in chronic lymphocytic leukemic cells, it is reported that no single biochemical mechanism is involved in chlorambucil resistance (310). This is probably the case for CLB-SPD in the human ovarian carcinoma cell lines in this study.

The results of this study suggest that, in human

ovarian carcinoma cell lines, there is no direct relationship between the activity of the polyamine uptake system, the affinity of CLB-SPD for the uptake system and their sensitivity to CLB-SPD. In addition, the amount of drug accumulated did not seem to affect the sensitivity. However, it was of interest, at least in these cells, that the reverse appeared to be true since the most sensitive cell line (PXN/94) had the least active uptake system for which the conjugate had a lowest affinity. If polyamine uptake/receptor binding did play a role in influencing the toxicity of CLB-SPD, it is possible that a lower affinity for the polyamine receptor may mean that more CLB-SPD actually reached the DNA since less intracellular binding may occur. In addition, intracellular polyamine levels may be low in the PXN/94 cells which could also result in less competition for DNA binding.

Due to the possible sequestration of CLB-SPD at sites distant from the DNA, which was apparent from results of chapters 5 and 6, an alternative mechanism of toxicity, not involving DNA, may be involved. For example, CLB-SPD may interact with the cell membrane in a similar manner as reported for Trenimon (218). A slower rate of transport may be conducive for toxicity, allowing CLB-SPD to exert its effects at the membrane level, possibly by alkylating the receptor or other membrane components. The longer the conjugate is in contact with the receptor, the greater the chance of alkylation occuring.

In summary, CLB-SPD toxicity was greater than chlorambucil in the PXN/94, SKOV-3, HX/62 and 41M human ovarian carcinoma cell lines, demonstrating that conjugation of chlorambucil to a polyamine carrier increased its toxicity. The importance of the kinetics of CLB-SPD uptake on the toxicity of the conjugate, however, was unclear since the relative contribution of passive uptake was certain. The toxicity of CLB-SPD did,

however, appear to be inversely proportional to the affinity of CLB-SPD for the uptake system which was is in direct contrast to the hypothesis that a high affinity for the uptake system should result in increased toxicity of polyamine-cytotoxic conjugates. It is likely that other factors play an important role in determining the sensitivity of the different cell lines to CLB-SPD,

CHAPTER 8 - Final Discussion

8.1 Introduction

The aim of this study was to attempt to target novel-polyamine cytotoxic conjugates to tumours via the polyamine uptake system present in tumour cells (56,64, 67,77,78,79,80,82). This has been attempted previously by several research groups (260,263,264,266), but with limited success, since the structural requirements of compounds to utilize the polyamine uptake system have not been fully considered in the design of novel conjugates.

In this study, several polyamine conjugates were investigated as to their suitability for use as targeted antitumour agents. Assays were optimized using a range of nitroimidazole-polyamine conjugates whilst awaiting the synthesis of more appropriate polyamine-cytotoxic conjugates. The assays included an initial assessment of the conjugates' abilities to inhibit spermidine uptake into tumour cells, which gave an indication of their affinities for the polyamine uptake system. Following this, the *in vitro* toxicity of the novel compounds was assessed, using inhibition of ^{3}H-thymidine incorporation to measure cytotoxicity. This was found to correlate well with the clonogenic assay. MGBG, a structural analogue of spermidine was used as a positive control compound in these studies.

8.2 Summary of results

The experiments with the nitroimidazole-polyamine conjugates demonstrated that conjugation of a nitroimidazole to a polyamine carrier resulted in an increase in the affinity of the compounds for the polyamine uptake system. Some enhancement in toxicity to tumour cells was also seen, especially in polyamine depleted cells. This was of interest since

nitroimidazoles are reported to normally only be directly cytotoxic under hypoxic conditions (311). It would appear that conjugation to a polyamine may increase the affinity of the nitroimidazoles for macromolecules such as DNA, RNA and proteins which are reported to be their target molecules (312),resulting in enhanced toxicity.

Based on the initial results of the studies with the nitroimidazole-conjugates and other polyamine analogues, and on information in the literature (77,97, 121,122), spermidine was chosen as the optimal carrier molecule. Spermidine can be substituted on the central nitrogen atom and still leave two primary amine groups to interact with the polyamine receptor which allows it to retain uptake characteristics similar to the polyamines. The original aim was to compare a range of polyamine-cytotoxic conjugates with small structural differences, for example different polyamine backbones or linker groups, to obtain the optimal compound. Although several polyamine-cytotoxic conjugates were synthesized, only one, a chlorambucil-spermidine conjugate (fig 3.2), was of sufficient purity to warrant further investigation. In this compound, chlorambucil was conjugated to the central nitrogen of spermidine via a spacer group which helped maintain the flexibility of the molecule to allow receptor and DNA interactions of the polyamine portion and the cytotoxic moiety. Due to a lack of other suitable compounds, the activity of CLB-SPD was studied in detail and its *in vitro* and *in vivo* activity compared to non-conjugated chlorambucil. In addition, an HPLC system was developed to allow the metabolism and tissue distribution of the two compounds to be compared.

In EATC, the cell line originally chosen for use in these studies because of its well documented polyamine uptake system (78,119,150,153), CLB-SPD had a high affinity for the uptake system, but was not very toxic unless cells were pretreated with DFMO to deplete polyamines. However, it is reported that, unless

polyamines are depleted, polyamine uptake in EATC is not very great (72). This could explain the observations with CLB-SPD. A further point to consider is that EATC were also quite resistant to chlorambucil itself.

Since EATC were not very sensitive to chlorambucil, the ADJ/PC6 plasmacytoma which is more sensitive to chlorambucil (290), was chosen for use in further studies. In this cell line, CLB-SPD was 30-fold more toxic than chlorambucil following a 1hr exposure. DFMO pretreatment increased this differential to 170 fold which was probably a combination of increased uptake (possibly via the polyamine uptake system) and also the effects of DFMO on the sensitivity of DNA to alkylating agents (160,161,162,165). Collaborative studies showed that CLB-SPD was 10,000 fold more reactive with naked DNA than chlorambucil, which was presumably due to the high affinity of the polyamine carrier for DNA (259). The spacer group between chlorambucil and the polyamine chain presumably also gave the molecule the flexibility required for chlorambucil to interact with DNA and form cross-links. The observation that the *in vitro* toxicity of CLB-SPD was only 30-fold greater than chlorambucil (compared to 10,000-fold greater DNA reactivity) suggests that endogenous polyamines and cations compete for DNA binding in cells.

Despite the promising *in vitro* activity, CLB-SPD was only 2-fold more active than chlorambucil *in vivo*. This required further investigation to determine whether the CLB-SPD conjugate was being metabolised *in vivo*, possibly by cleavage of the amide linkage, releasing free chlorambucil. This was investigated using a specially developed HPLC system and it was shown that cleavage did not occcur and that CLB-SPD was not acting as a prodrug.

The levels of CLB-SPD and chlorambucil in tumour and normal tissues were then compared to confirm that the conjugate was getting into the tumour. Drug delivery was satisfactory with approximately 10-fold higher levels of

CLB-SPD than chlorambucil being achieved in tumour tissue. The enhanced CLB-SPD uptake, however, was not tumour specific but seen in all tissues. In addition, the observation that CLB-SPD remained in tissues for several days suggests that the conjugate may have become sequestered at sights distant from its DNA target, possibly by intracellular binding. Paulus *et al.* also report that sequestration of spermidine occurs as a result of it binding strongly to anionic sites in the cell such as ribosomes and membranes (313).

In addition to sequestration, the observation that unchanged CLB-SPD was present in tissues several days after administration suggests that the chloroethyl groups of CLB-SPD may be protected from hydrolysis. This could possibly be by the polyamine chain itself which could shield the chloroethyl groups from nucleophiles, including water and DNA. If sequestration of CLB-SPD was to occur immediately on entry to the cells, this would explain why, despite achieving higher levels in the tumour, it was only 2-fold more active than chlorambucil *in vivo.*

An interesting observation made with an impure batch of CLB-SPD which contained approximately 15% of a dichlorambucil-spermidine conjugate, was that it was several fold more active both *in vitro* and *in vivo* than pure CLB-SPD. A possible explanation for this could be that the diconjugate was sequestered less since it is less positively charged due to substitution of an extra N atom. In addition, the polyamine chain cannot protect the chloroethyl groups which means they are more likely to react with cellular nucleophiles. A further consideration is that the dichlorambucil conjugate has the potential to undergo 4 alkylation reactions with DNA, which may produce a more damaging lesion. Attempts to synthesize pure dichlorambucil-spermidine are currently underway to allow further investigation of this compound.

The tissue distribution studies showed that CLB-

SPD did not accumulate specifically in tumour tissue but
accumulated in all tissues. Levels obtained in kidney
and liver were particularly high and remained elevated
for several days. The tissue distribution of CLB-SPD was
compared to MGBG to see whether the two compounds had a
similar distribution pattern, which would have been
expected if they both utilized the polyamine uptake
system. However, their tissue distribution was quite
different with MGBG accumulating to a large extent
in the small intestine but not in the kidney. A salient
point to consider was the observation that despite
reaching high levels in the kidney, CLB-SPD did not
demonstrate toxicity to this tissue, whereas MGBG did.

Prior administration of DFMO to deplete
polyamines *in vivo* is reported to result in an increase
in the uptake of polyamines and polyamine analogues into
tumours and the small intestine (74,146,147,148,188,303).
In this study, this was seen for MGBG but not for CLB-
SPD. These results suggest that CLB-SPD does not act
like a polyamine with regards to its tissue uptake and
distribution and that the polyamine uptake system may not
be critical for determining its uptake. Further evidence
for this was provided by the observation that it was not
possible to alter the tissue distribution of CLB-SPD
using osmotic minipumps or continuous infusion which
should have improved the selectivity to tissues with a
polyamine uptake system. In contrast, MGBG uptake into
tumour, lung and small intestine was increased relative
to other tissues when osmotic minipumps were used to
administer the compound.

The limitations of the HPLC method used to detect
CLB-SPD, however, mean that covalently bound CLB-SPD was
not detectable and that true levels could, in fact, be
higher than shown. For example, Diam-3, a polyamine
conjugate, becomes extensively bound and is not released
from tissues by TCA precipitation (265). In addition,
the method does not allow for the fact that small

increases in accumulation may occur in certain cell types within a tissue which may be masked by high levels in the surrounding cells. A poor blood supply to the tumour could also interfere with CLB-SPD accumulation.

The polyamine uptake system, therefore, did not appear to have a major influence on the accumulation of CLB-SPD. It was decided to see whether the *in vitro* toxicity of CLB-SPD to a panel of human ovarian carcinoma cell lines had any relationship to the activity of the polyamine uptake system in the different cells or the affinity of the CLB-SPD conjugate for the uptake system. It was originally proposed that cells with a very active uptake system (ie. low Km and high Vmax) should be more susceptible to the polyamine-cytotoxic conjugates, especially if the conjugates had a high affinity for the polyamine uptake system. However, no direct relationship was found. In fact the sensitivity of the different cell lines actually appeared to be inversely proportional to the Ki values obtained for the conjugate. Other factors, such as their sensitivity to alkylating agents (230,234, 235,236,310) may have dictated the sensitivity of some of the cell lines to CLB-SPD although this was probably not so for PXN/94 cells which show varying responses to alkylating agents. CLB-SPD, however, was more toxic than chlorambucil in all four human ovarian carcinoma cell lines.

8.3 Is spermidine the optimal carrier molecule ?

The results of these studies clearly show that conjugation of a cytotoxic to a spermidine carrier resulted in increased tumour cell toxicity in a number of tumour models. This was probably due to targeting of the DNA itself rather than effects of the polyamine uptake system. A similar effect is reported for bleomycin, where the polyamine moieties in the compound are thought to be responsible for its DNA interaction (121). The apparent sequestration of CLB-SPD at sites distant from the DNA limit the usefulness of the conjugate as a

targeted antitumour agent since it would presumably
cancel out any subtle differences in cellular uptake that
may occur. Porter and Sufrin (121) suggested that the
inactivity of a number of polyamine-cytotoxic conjugates
they synthesized was due to the use of an N^4 acyl linkage
rather than an alkyl linkage in the molecules. They
suggested that the poor activity was due to poor uptake
of the compounds. However, the results of this study
suggest that good uptake is not necessarily indicative of
good activity and that intracellular binding may play an
important role in reducing the amount of drug reaching
the DNA target. One would envisage that for a compound to
be a substrate of the polyamine uptake system and not be
too highly bound intracellularly, a fine balance
regarding the charge on the molecule would have to exist.
If sequestration of the conjugates cannot be overcome, it
may be possible to use conjugates which have activity at
the sites of sequestration,for example cell or nuclear
membranes.

The majority of reports in the literature, in
which polyamine uptake has been compared directly in
normal and tumour tissue/cells, have used putrescine as
the model polyamine (66,67,145,146). It is possible that
spermidine uptake may not be as enhanced as putrescine in
tumours. In fact, distinct differences in the uptake
systems for these two compounds have emerged (see chapter
1). A further interesting point to consider is the
observation that, despite the apparent unsuitability of
diamines as the carrier molecules, two very successful
conjugates to date have in fact been aziridinyl
putrescine (266) and Diam 3 (265), both of which have
diamine carriers. The use of a spermidine carrier for
the active groups in these compounds has not been
reported, but a comparison of the two carriers would be
useful.

8.4 Future work

A number of further studies could be carried out with the CLB-SPD conjugate. Firstly it would be useful to see whether the promising *in vitro* activity demonstrated against the PXN-94 human ovarian carcinoma cell line can be reproduced *in vivo* against a human tumour xenograft. Also, the high levels of conjugate seen in the kidney could indicate that it may possess activity against kidney tumours. The CNS toxicity of CLB-SPD, together with reports in the literature that polyamines can cross the blood brain barrier (26,27), indicate that CLB-SPD may also cross the blood brain barrier to some extent. Therefore its activity against kidney and brain tumours could also be investigated.

It has been reported that nitroimidazoles bring about an increase in hypoxic tumour cell kill when used in combination with alkylating agents such as melphalan (311,314). This is thought to be due to a combination of altered metabolism of the alkylating agent, temperature effects and also an enhancement of DNA interstrand cross-linking which may be due to decreased repair (297,311, 314). It would be of interest to see whether any of the nitroimidazole-polyamine conjugates possessed these characteristics, and whether perhaps they were even better chemosensitizers than the non-conjugated parent compounds.

In addition to further antitumour testing, it would be useful to measure the formation of DNA interstrand crosslinks, single strand breaks and DNA-protein crosslinks by chlorambucil and CLB-SPD and their repair by alkaline elution. Since DNA interstrand crosslinks are assumed to be the cytotoxic lesion produced by simple nitrogen mustards such as chlorambucil (201,202,203,204,205,206), comparisons could be made at equitoxic concentrations to determine the relative importance of crosslinking to the cytotoxicity of CLB-

SPD. This would reveal whether the DNA lesions caused by CLB-SPD are of similar potency to those of chlorambucil. In addition, it would be interesting to determine whether the DNA sequence selectivity of chlorambucil for N^7guanine in GC rich areas is altered by its conjugation to spermidine. The cell cycle specificity of the compounds could also be compared using fluorescence activated cell sorting (FACS) analysis.

It has been reported that the mechanism of action of alkylating agents in some sensitive cells may involve apoptosis (315). Following exposure to nitrogen mustard, sensitive cells were unable to complete S phase while cross-links were being removed. This resulted in a delay in S phase which made them susceptible to apoptosis (315). The observation that apoptotic bodies were present in many tissues following the administration of chlorambucil and CLB-SPD to mice suggests that these compounds may also cause apoptosis. This clearly warrants further investigation.

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