THE SYNTHESIS AND ANTITUMOUR EVALUATION OF METHYLAMINO COMPOUNDS

by

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To my parents

The synthesis and antitumour evaluation of methylamino compounds

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Submitted for the degree of Doctor of Philosophy, 1983, in the University of Aston in Birmingham.

The history and development of antitumour agents which require N-methyl moieties for optimum activity is reviewed. Their metabolism as well as the biological and chemical reactivity of their metabolites is discussed together with hypotheses concerning their mechanism of antineoplastic action.

Analogues of the antitumour agent hexamethylmelamine [HMM; 2,4,6-tris(dimethylamino)-s-triazine] were synthesized and characterised including a series of novel 3-aryl-s-triazolo [4,3-a]-1,3,5-triazines and 2-aryl-s-triazolo[2,3-a] -1,3,5-triazines whose spectroscopic properties were investigated.

The chemosensitivity of the M5076 reticulum cell sarcoma in the mouse was examined and the analogues of HMM assayed against this murine tumour system. The *in vitro* cytotoxicity of these compounds against PC6 plasmacytoma cells was shown to correlate with the extent to which they were metabolized *in vitro* while their *in vivo* antitumour activity was shown to correlate with their *in vivo* biotransformation to formaldehyde precursors. These results suggest that for an analogue of HMM to possess antitumour activity it must readily undergo oxidative metabolism of the N-methyl group *in vivo*.

N-Methylformamide (NMF), another N-methyl containing antitumour agent, was shown to be active against the M5076 sarcoma and Sarcoma 180 models. A structure-activity study demonstrated the requirements of both its formyl and N-methyl moieties for optimal antitumour activity. The drug was shown to possess hepatotoxic potential but was not myelosuppressive in mice. Combinations of NMF with either cyclophosphamide or HMM were investigated. The combination of NMF with cyclophosphamide elicited an improved antitumour response against the M5076 sarcoma in mice compared to that obtained with the single agents, while neither the hepatotoxicity caused by NMF nor the myelosuppression caused by cyclophosphamide were exacerbated.

Key words: Hexamethylmelamine, N-Methylformamide.

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ABBREVIATIONS

HMM	Hexamethylmelamine	2,4,6-Tris(dimethylamino)-*s*-triazine
PMM	Pentamethylmelamine	2,4-Bis(dimethylamino)-6-methylamino-*s*-triazine
TMM	N^2,N^2,N^4,N^4-Tetramethylmelamine	2-Amino-4,6-bis (dimethylamino)-*s*-triazine
TriMM	N^2,N^4,N^6-Trimethylmelamine	2,4,6-Tris(methylamino)-*s*-triazine
M	Melamine	2,4,6-Triamino-*s*-triazine
HMPMM	Hydroxymethylpenta-methylmelamine	{N- 4,6-Bis(dimethylamino)-*s*-triazin-2-yl - methylamino}-methanol

ABDT	2-Azido-4,6-bis(dimethylamino)-*s*-triazine
CBDT	2-Chloro-4,6-bis(dimethylamino)-*s*-triazine
HBDT	2,4-Bis(dimethylamino)-6-hydrazino-*s*-triazine
DTIC	5-(3,3-dimethyltriazen-1-yl)imidazole-4-carboxamide
MTIC	5-(3-monomethyltriazen-1-yl)imidazole-4-carboxamide
NMF	N-Methylformamide
F	Formamide
NEF	N-Ethylformamide
DMF	NN-Dimethylformamide
HMF	N-Hydroxymethylformamide
HMMF	N-Hydroxymethyl-N-methylformamide
BCNU	1,3-Bis(2-chloroethyl)-1-nitrosourea
CCNU	1-(2-Chloroethyl)-3-cyclohexyl-1-nitrosourea
MOPP	Mechlorethamine/Vincristine(oncovin)/procarbazine/prednisone

CVPP	Cyclophosphamide/vinblastine/procarbazine/prednisone
Hexa-CAF	Hexamethylmelamine/methotrexate/5-fluorouracil/ cyclophosphamide
CHAP	Hexamethylmelamine/adriamycin/cis-platinum/ cyclophosphamide
GSH	Glutathione
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
NADH	Nicotinamide adenine dinucleotide (reduced form)
B.S.A.	Bovine serum albumin
G6P	Glucose-6-phosphate
G6P dehydrogenase	Glucose-6-phosphate dehydrogenase
SDH	Sorbitol dehydrogenase
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
WBC	White blood cells
AUC	Area under the plasma concentration-time curve
IST	Increase in survival time
i.p.	intraperitoneal
p.o.	per os
i.v.	intravenous
s.c	subcutaneous
i.m.	intramuscular
n.m.r.	nuclear magnetic resonance
i.r.	infra red
u.v.	ultra violet
h.p.l.c.	high performance liquid chromatography
g.l.c.m.s.	gas-liquid chromatography mass spectrometry
s.d.	standard deviation

LD_{10}	Lethal dose (10%)
LD_{50}	Lethal dose (50%)
M5	M5076
PC6	ADJ/PC6A
N.C.I.	National Cancer Institute
C.R.C.	Cancer Research Campaign

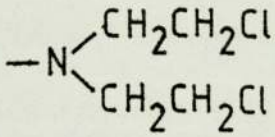
SECTION 1

INTRODUCTION

CHAPTER 1 The role of the N-methylamino group in cancer chemotherapy

1.1 Introduction

Since the advent of modern cancer chemotherapy in 1942, with the first clinical trial of nitrogen mustard in a patient with lymphosarcoma[1], it has been recognised that the presence of certain chemical moieties within a structure may confer antitumour properties to that compound. Notable amongst these are the N,N-bis(2-chloroethyl)amino (1) and aziridinyl (2) functionalities.



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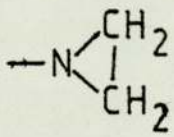
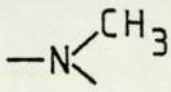


(2)

Representative of clinically employed agents containing the former include nitrogen mustard (3), melphalan (4), cyclophosphamide (5) and chlorambucil (6); and of the latter, triethylenemelamine (7) and triethylenethiophosphoramide (8).

Recently the presence of an N-methylamino group (9) within a number of structures has been recognised to be of crucial importance for their antitumour activity.[2,3]



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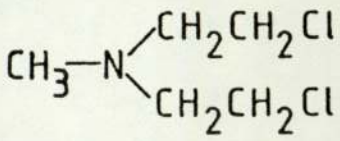

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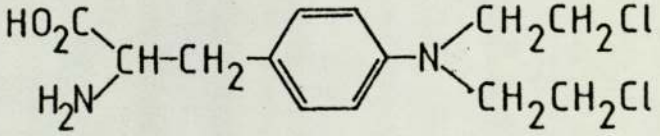

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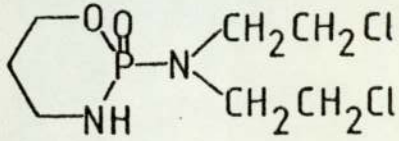

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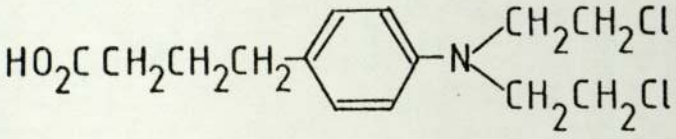

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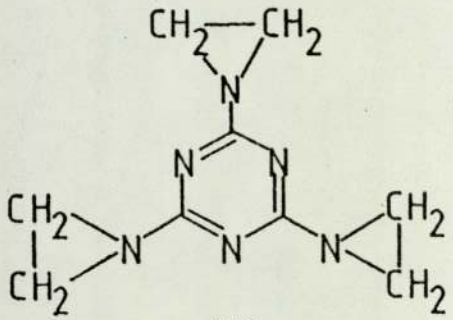

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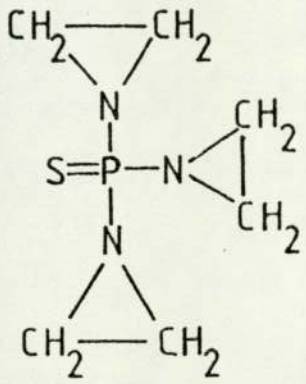

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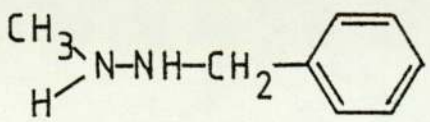
Replacement of this group renders the compounds inactive and thus it may represent a new antitumour micro-moiety. Agents of this type include the following; procarbazine (10), 5-(3,3-dimethyltriazen-1-yl)imidazole-4-carboxamide (DTIC;11), hexamethylmelamine (12) and N-methylformamide (13).

The mechanisms of action of all of these compounds are unknown and therefore it is not possible to say whether the mechanistic reason for the requirement of the N-methyl group is the same in all cases.

This thesis is concerned with the chemistry, antitumour activity and mode of action of two of these agents, hexamethylmelamine (12) and N-methylformamide (13). Since certain features of the biochemistry and biology of the N-methylamino group within the other structures (10,11) are of relevance to an understanding of how these two agents might act, they will first be reviewed.

1.2. Procarbazine

In 1963, a search for derivatives of 1-methyl-2-benzylhydrazine (14) with antitumour activity led to the discovery of procarbazine (10)[4,5]. Procarbazine [1-methyl-2-(4-isopropylcarbamoyl)benzylhydrazine hydrochloride] has found clinical use mainly in the treatment of Hodgkin's disease[6-10], but it is also of benefit in the treatment of non-Hodgkin's lymphomas[11], small cell carcinoma of the



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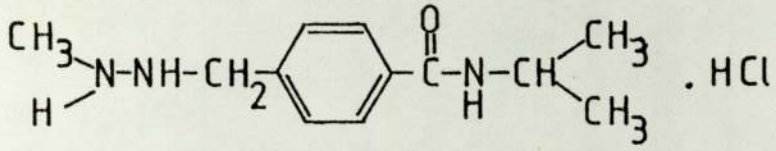

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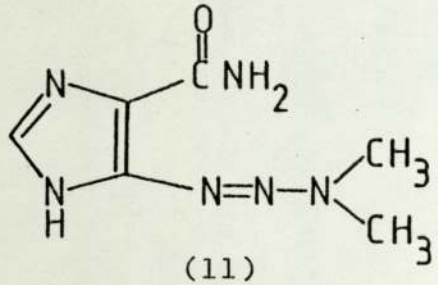

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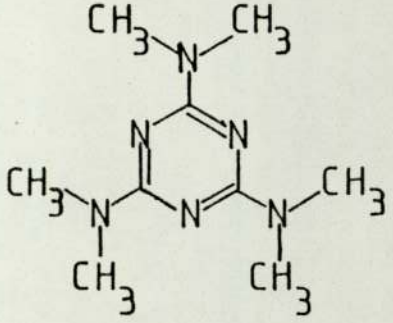

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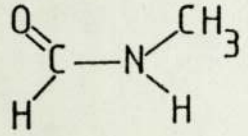


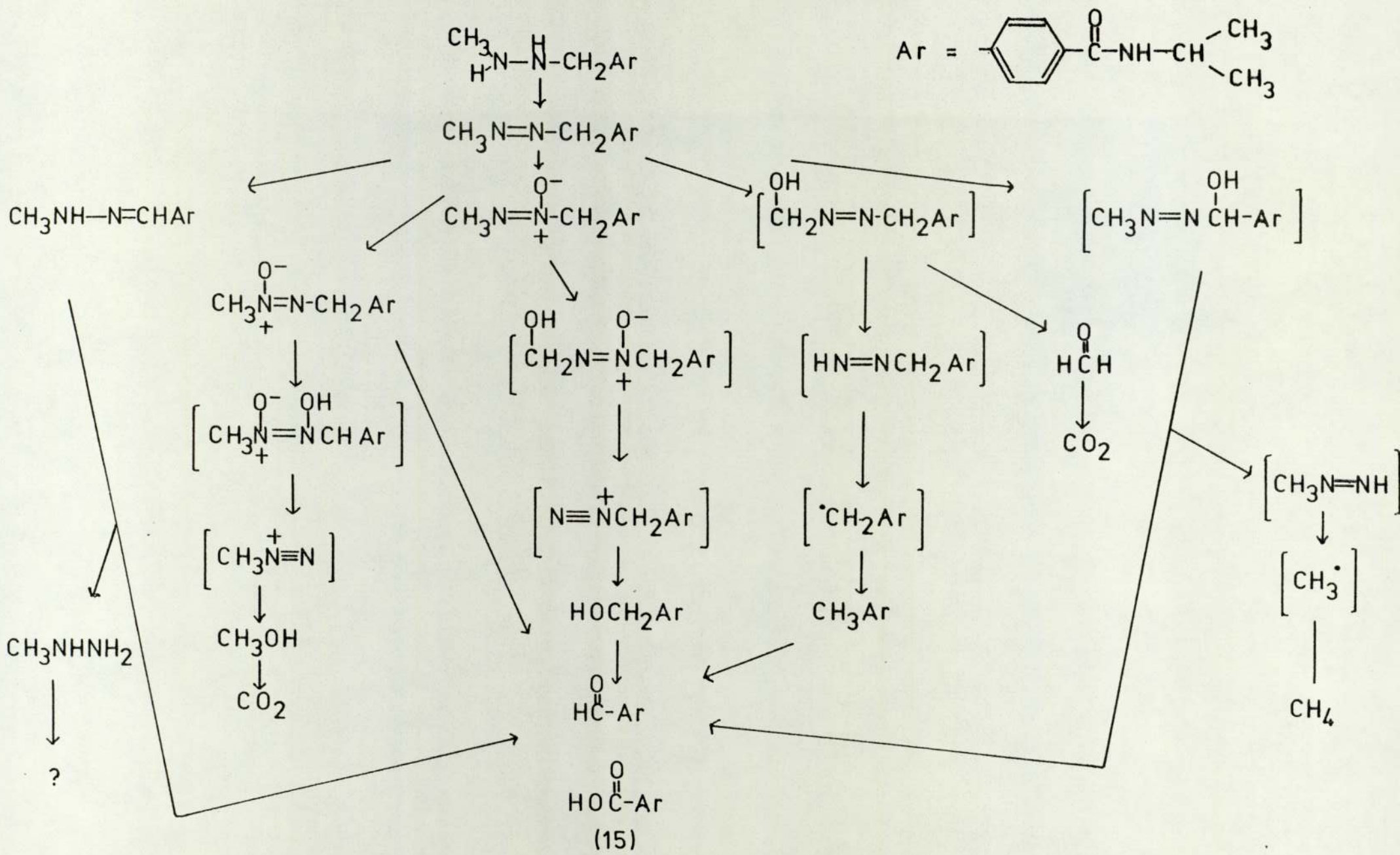
(13)

lung,[12] malignant melanoma[7,13], and glioblastomas[14]. It is now most commonly employed as a component of either the MOPP[15] or CVPP[16] combinations.

The mechanism of action of the compound is unknown and its metabolism complex. Studies by Weinkam *et al.*[17] and others[18-26] indicate at least five pathways by which the drug may be metabolised *in vivo*, pathways which lead to chemical inactivation, alkylation reactions and free radical formation (scheme 1.1). It is unknown which of the many species is (or are) responsible for antitumour activity. What is clear, and moreover was demonstrated soon after the discovery of the drug, is the absolute requirement of the methylhydrazino group for antitumour activity. Replacing the methyl with a H atom[4,27,20] or ethyl[4,20] group renders the molecule biologically inactive.

Labelling the N-methyl group with ^{14}C has allowed elucidation of its metabolic fate[20,28,29]. Expiration by rats treated with $^{14}CH_3$-labelled procarbazine has indicated both $^{14}CO_2$[21,30-32] and CH_4[23,33,34] formation in a CO_2/CH_4 ratio of about 2.[23,34] About 25% of the administered radioactivity is expired[35] and this is of the same order of magnitude as the amount of 4-isopropylcarbamoylbenzoic acid (15) (the final ring-containing metabolite) produced.

The rate of CO_2 production was enhanced by the pretreatment of rats with phenobarbitone or 3-methylcholanthrene[21,22,30]



Scheme 1.1 Possible metabolic pathways for procarbazine.

and markedly lowered by SKF 525A[22], all known modifiers of oxidative microsomal enzymes; this implies involvement of cytochrome P450 enzymes in the rate determining metabolic steps. Kreis [20] has demonstrated, using tritiated ^{14}C-methyl labelled procarbazine, that the intact methyl group can transmethylate tRNA bases, pricipally methylating the N(7) position of guanine and has suggested that this might play a role in the carcinostatic and carcinogenic activity of the drug.

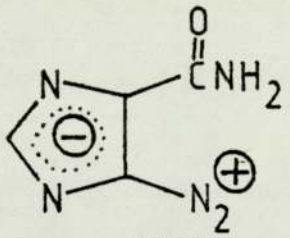
Metabolic activation appears to be a requirement both for the cytotoxicity[36] and mutagenicity[37] of procarbazine and it has been suggested that the alkylating species produced might be responsible for the antitumour activity of the drug. If this is so, then it is difficult to explain the inactivity of the N-ethyl analogue since it is likely that many of the homologous species would be evolved during its metabolism. Qualitative differences however may exist between the two respective metabolic routes such as a crucial pathway that is open to one and not the other alkyl substrate. Alternatively quantitative differences may be important such as in the relative reaction rates and differing stabilites of metabolites. The metabolism of the N-ethyl analogue has not yet been reported and until so the reasons for its inactivity can only remain conjectural.

1.3 Triazenes

The role of the N-methyl group within the triazene structure has been under discussion for several years. Whereas some

authors have described its presence as an 'absolute requirement' for activity[38], others have questioned the need for it at all.[39] The arguments are reviewed in the following pages.

In 1961, Shealy et al.[40] reported the synthesis of 5-diazoimidazole-4-carboxamide (16) from 5-aminoimidazole-4-carboxamide and its potential antitumour activity. Due to its instability in aqueous solutions, di- and mono-substituted derivatives were tested and 5-(3,3-dimethyltriazen-1-yl)imidazole-4-carboxamide (DIC,DTIC:11) was selected for clinical trial.[41]



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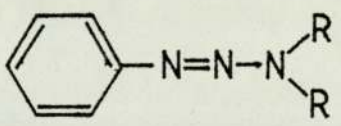
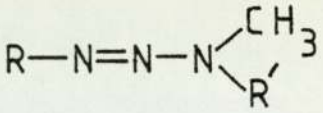


(17)

It has since demonstrated objective clinical activity against malignant melanoma.[42-48] Several years previously in 1955, 3,3-dimethyl-1-phenyltriazene (17;R=CH_3) was shown by Clarke et al. to inhibit the Sarcoma 180.[49] Within the same report, the diethyl analogue (17;R=C_2H_5) was reported to be inactive. Thus as with procarbazine the preference of the N-methyl group over that of the N-ethyl group was observed at a very early stage of research into the triazenes.

A vast number of triazenes have now been synthesized and tested for antitumour activity. Wilman et al. have suggested that the basic structure for activity may be summarized as in (18).[50]

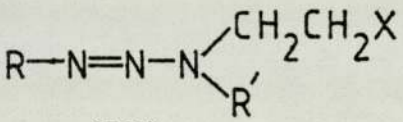


(18)

where R = aryl or heteroaryl group ('carrier group')

R′= a group more susceptible to metabolism than the N-methyl group

Although this structure-activity relationship appears to be true in the case of studies with the TLX5 lymphoma[50-52] and is also valid for most compounds tested on the L1210 and P388 murine leukaemias, there are important exceptions. Firstly 2-haloalkyl groups may replace the N-methyl group, thus compounds such as (19) are active.[53,54]



(19)

where X = Cl, F

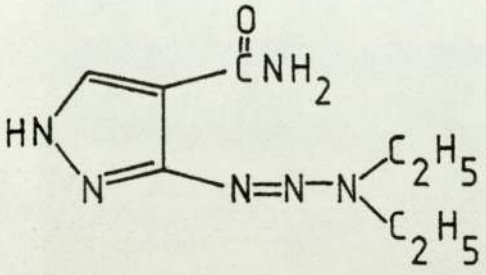
R = aryl or heteroaryl group

R' = H, alkyl or 2-haloalkyl group

It is not known whether these compounds work through mechanisms analogous to the methyltriazenes or not.

Secondly the National Cancer Institute (N.C.I.) have reported several compounds, which, although containing no

methyl group, still retain weak but significant activity against the L1210 leukaemia e.g. (20) and (21).[39]



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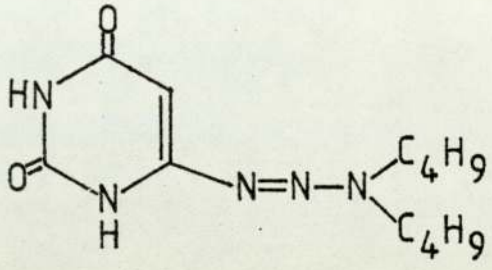

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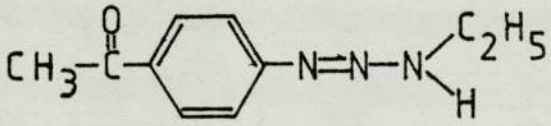
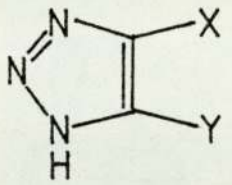
More recently a 1-aryl-3-ethyltriazene (22) has been reported to be active against the P388 leukaemia.[55] It is however inactive towards the TLX5 lymphoma.[56]



(22)

A great deal of variety within the carrying structure R in (18) is permitted. Shealy[53] has demonstrated using the L1210 leukaemia that several heteroaryl groups are possible for R without ensuing loss of activity, including the following (23) - (26).



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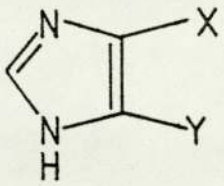

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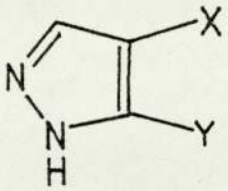

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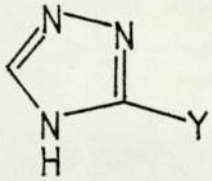
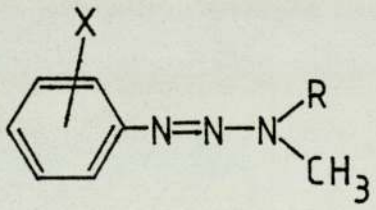


(26)

where X = $CONH_2$ e.t.c.

Y = $-N=N-N(CH_3)_2$

Alternatively a substituted phenyl group (27) may be inserted for Rin(18). Connors et al. have shown that varying the aromatic substituent (X) in (27:R=CH_3), i.e. by using electron-withdrawing or electron-donating groups in the o-,m- or p- positions, does not greatly affect the anti-tumour properties of the molecule against the TLX5 lymphoma.[52] Although this moiety of the structure appears relatively unimportant it will determine the stability of the intact molecule and its subsequent metabolic and pharmocokinetic profile.



(27)

In 1976, Connors et al. proposed that for a 1-phenyl-3,3-dialkyltriazene to be active it should undergo metabolism to

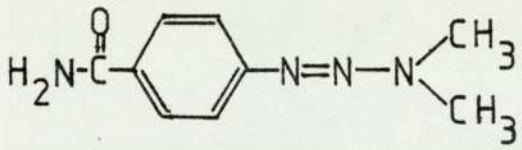
a 1-phenyl-3-methyltriazene (27:R=H) thus implying that the compound should initially contain at least one N-methyl group.[52] In addition the group R should be susceptible to metabolism before the methyl group. His hypothesis was based on the following observations:

i) compounds containing a group R which is preferentially dealkylated before the methyl group (e.g. R=ethyl or propyl) produce monomethyltriazenes *in vitro* and are active *in vivo* against the TLX5 lymphoma.

ii) compounds containing a group R which cannot be dealkylated (e.g. R=t-butyl) do not produce monomethyltriazenes *in vitro* and are inactive *in vivo*.

iii) compounds containing no methyl group are inactive *in vivo* although they often undergo efficient dealkylation *in vitro* (e.g. 1-phenyl-3,3-diethyltriazene or 1-phenyl-3,3-diisopropyltriazene).

Further evidence supporting the hypothesis that the monomethyltriazene might be the active metabolite comes from a comparison of the cytotoxicites of a 1-aryl-3,3-dimethyltriazene ('dimethyltriazene') (28) with its corresponding monomethyl derivative against TLX5 lymphoma cells *in vitro*.[52] Whereas the dimethyltriazene is non-toxic *per se*, activation with liver microsomes, cofactors and oxygenation produces a ten-fold increase in toxicity. On the other hand,



(28)

the monomethyltriazene is toxic without activation and incubation with liver microsomes renders it less cytotoxic to these cells. The conclusion is that the dimethyltriazene can be activated by liver supernatant to the monomethyltriazene but further metabolism results in deactivation. Since the monomethyltriazene is presumably the most cytotoxic species in the system, then it is assumed to be the most likely 'active metabolite'. However these experiments cannot take into account the differing stabilities and transport abilites of the various species since it is the level of the drug at the tumour site and not just relative cytotoxicity that will determine antitumour activity *in vivo*.

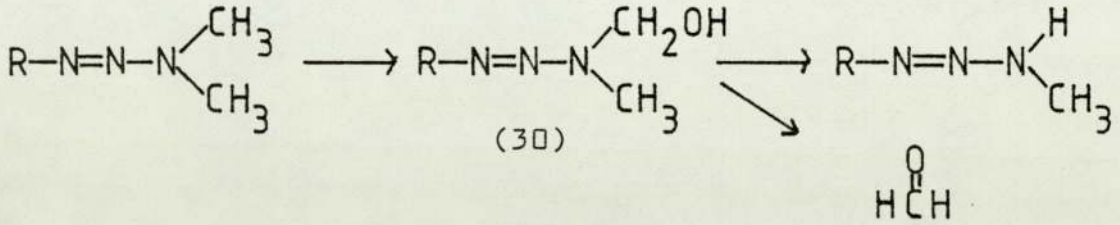
Horton *et al.* have demonstrated with several dimethyltriazenes (27; R=CH_3; X=4-cyano, 4-nitro, 4-acetyl) that the amounts of their respective monomethyltriazenes generated upon microsomal activation *in vitro* were insufficient to account for the observed cytotoxicity to TLX5 cells;[57] this suggests another species is probably responsible for antitumour activity.

This important requirement for the N-methyl group in most cases is very difficult to explain. One hypothesis for the mechanism of antitumour action of the dimethyltriazenes is through their ability to alkylate DNA.[58] Gescher *et al.*[38]

have pointed out that this parallels the proposed mechanism by which triazenes are thought to exert their carcinogenicity;[59] the dimethyltriazene metabolically generates the monomethyltriazene which is known to be a powerful methylating agent.[60] However diethyltriazenes are able to generate monoethyltriazenes,[59] at least *in vitro*, which are again known to be carcinogenic and alkylate DNA and yet are completely inactive as antitumour agents against the TLX5 lymphoma. Also the host toxicity of diethyltriazenes is very similar to that of dimethyltriazenes suggesting production of equal amounts of cytotoxic species.[51,56]

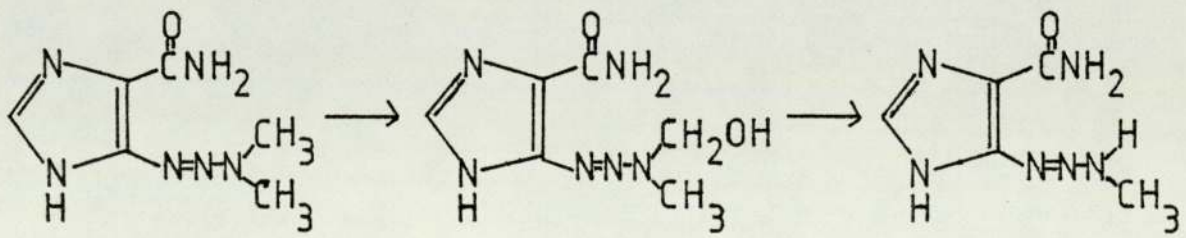
Hickman[2] and Gescher *et al.*[38] have suggested that although the monomethyltriazenes are highly cytotoxic, they may not be the active metabolites. Comparisons of monomethyl- and monoethyltriazenes (18; R= 4-tolyl) against TLX5 cells show them to be of similar cytotoxicity.[38] Also monomethyltriazenes are equally cytotoxic *in vitro* to TLX5 cells which are either sensitive or resistant to dimethyltriazenes suggesting the cytotoxicity to be non-selective.[2,38] Paradoxically in the same study a monomethyltriazene (18; R= 4-carbomethoxyphenyl) was shown to be inactive against the resistant cell line *in vivo* whilst showing activity against the sensitive cell line.[38] This result contrasts with the finding that MTIC (29), the monomethyl analogue of DTIC (11) is equally active *in vivo* towards cell lines of the L1210 resistant and sensitive to DTIC.[54]

Another possible candidate for 'active metabolite' is the N-hydroxymethyl-N-methyltriazene (30) - 'a carbinolamine'.[2,38] This is the proposed intermediate in the metabolism of the dimethyltriazene to the monomethyltriazene (scheme 1.2). Originally these molecules were thought to be too short lived to be of importance. However several such carbinolamines



Scheme 1.2 Metabolism of the dimethyltriazenes

have now been synthesized and found to be relatively stable species.[61,62] Hickman has shown one of these (30; R= 4-carboxymethylphenyl) to be more potent than the corresponding monomethyltriazene both <u>in vivo</u> and <u>in vitro</u> against the TLX5 lymphoma. The hydroxymethyl intermediate (31) (scheme 1.3) of the clinically used triazene, DTIC, has now been tentatively identified by t.l.c. as a metabolite in rat urine and shown to



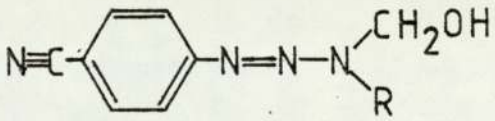
DTIC (11) (31) MTIC (29)

Scheme 1.3 Metabolism of DTIC

be more stable than MTIC both in polar solvents and under conditions of chromatography.[63] Kolar has suggested that this species might act as a transport form of the DTIC derived methylating agent.[63] He has further suggested that it may directly participate in tumour inhibition by covalent binding to, or intercalation with, nucleic acids.

The hydroxymethyl intermediate may act as a transport form of formaldehyde and release of this cytotoxic moiety at the tumour site may be responsible for antitumour activity. On the other hand the N-ethyl metabolite, N-(1-hydroxyethyl)-1-alkyltriazene, releases acetaldehyde and this may represent a very much less effective antitumour species. Evidence that formaldehyde does indeed have tumour inhibitory properties *in vivo* will be presented later in this thesis.

However the carbinolamine, in similar fashion to the monomethyltriazene, is unable to discriminate *in vitro* between TLX5 cell lines sensitive and resistant to the dimethyltriazenes.[64] In addition, an N-ethyl-N-hydroxymethyltriazene (32; R= ethyl) has been shown to be inactive towards the TLX5 lymphoma whereas its N-methyl analogue (32; R= methyl) retained activity suggesting that the carbinolamines *per se* are not the active species. [64]

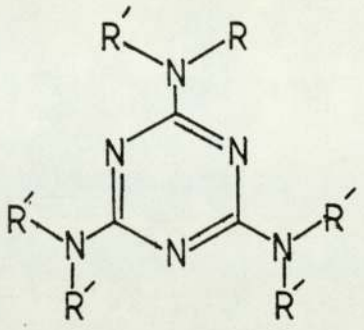


(32)

In conclusion, therefore, although the requirement for the N-methyl group has been very much more carefully studied for the triazenes than for procarbazine, the explanation for the preference of the N-methyl group over the N-ethyl group for antitumour activity remains just as elusive.

1.4 Hexamethylmelamine

Hexamethylmelamine [2,4,6-tris(dimethylamino)-s-triazine] (HMM; 12) has shown consistent clinical activity against a wide variety of solid tumours including small (oat) cell carcinomas of the lung,[65,66] ovarian carcinomas[67,68] and breast tumours.[69-71] Bilharzial bladder cancer,[72,73] cervical cancer[74] and lymphomas[69,74,75] have all shown favourable responses. Since the primary toxic effects of HMM are gastrointestinal and neurological,[76] it has found widespread use in combination chemotherapy alongside myelotoxic agents. It is currently in use as a constituent of some of the most promising combinations for the treatment of ovarian tumours including Hexa-CAF,[77-81] and CHAP.[82-84] Recently pentamethylmelamine (PMM; 33), the desmethyl analogue of HMM, which is soluble enough for intravenous infusion has entered several phase 1 clinical trials in an attempt to circumvent the gastrointestinal toxicity.[85-90]



(12) R = CH_3 , R′ = CH_3

(33) R = H , R′ = CH_3

(34) R = H , R′ = H

Initial indications are not promising and show that PMM is no less of an emetic than HMM.

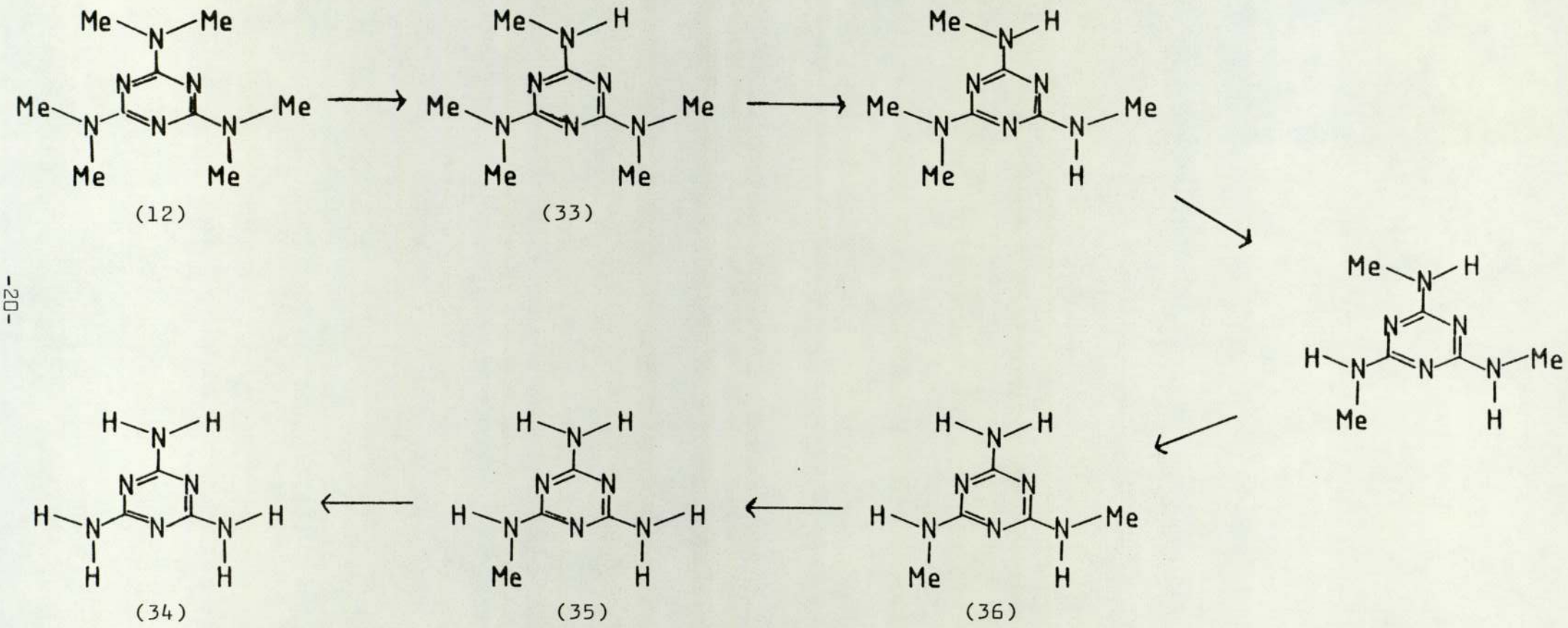
The importance of the N-methyl group within the structure was demonstrated in 1975 by Lake et al.[91] using the murine Sarcoma 180 and Lewis Lung carcinoma tumours. Increasing the number of methyl groups from one as in monomethylmelamine to six as in HMM resulted in a gradual increase in both toxicity and antitumour activity. Since the two parameters changed at about the same rate the therapeutic index remained constant. Melamine (34), however, with no methyl group, was without activity and thus the suggestion was advanced that the presence of a methyl group and not the number of groups was the determining factor for antitumour activity within these compounds.

Early studies on the metabolism and pharmacokinetics of HMM established that the drug was extensively metabolised in both

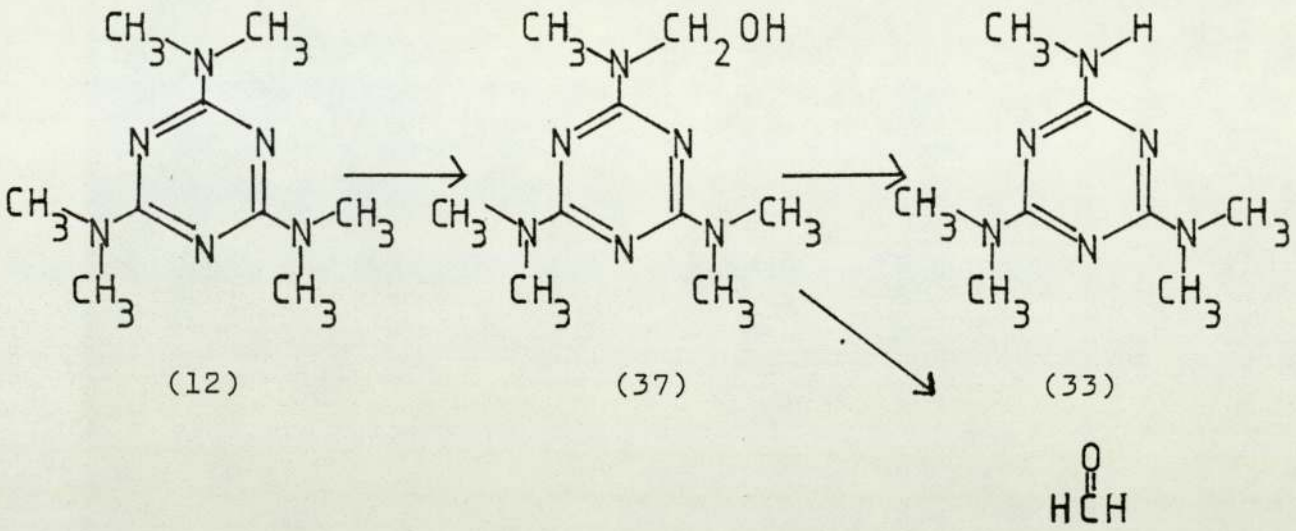
rats and man.[92,93] Oral administration of ^{14}C-methyl-HMM to patients was followed by the appearance of respiratory $^{14}CO_2$ (9% of radioactivity within 6h) and labelled metabolites in the urine (29% in 72h).[92] No unchanged HMM was detected and all urinary metabolites were identified as desmethyl analogues of HMM. From the pattern of metabolites it was possible to identify the major route of demethylation and this is shown in scheme 1.4. Minute quantities of the other methylmelamines, N^2,N^2,N^4,N^4-tetra-, N^2,N^2,N^4-tri- and N^2,N^2-dimethylmelamine were also found.

In rats the same metabolic pathway was found with 64% of the initial dose of HMM being excreted as metabolites with an intact triazine ring.[92] Use of the ^{14}C-ring-labelled drug established that no significant cleavage of the triazine ring occurred and N^2,N^4-dimethylmelamine (36) and monomethylmelamine (35) were the major urinary metabolites of HMM.[93] PMM shares a similar metabolic fate.[94-97]

There is strong evidence that HMM requires metabolic activation for its antitumour activity.[98] Whereas it is cytotoxic <u>in vitro</u> only after very prolonged exposures to cells,[99,100] one of its major <u>in vitro</u> metabolites N-hydroxymethylpentamethylmelamine (HMPMM: 37) - a 'carbinolamine' - is a very much more cytotoxic species and does not require activation.[99,100] It is an intermediate in the oxidative N-demethylation as shown in scheme 1.5.



Scheme 1.4 <u>In vivo</u> metabolism of HMM



(12) (37) (33)

Scheme 1.5 Oxidative N-demethylation of HMM

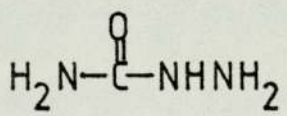
Although the intermediate (37) has been identified as a metabolite *in vitro* by h.p.l.c. and g.l.c.m.s. studies after derivatisation[101] it has not yet been identified *in vivo*. Gescher *et al.* have suggested that once generated it may conceivably undergo rapid conjugative metabolism and probably extensive biliary excretion.[101]

The metabolism of HMM can occur both in the liver[98,101-103] and extrahepatically.[103-105] Irreversible binding of labelled metabolites of HMM has been demonstrated both *in vivo* and *in vitro* to liver and ovarian tumour macromolecules.[103]

Whereas ^{14}C-ring-labelled HMM in the absence of cofactors had only a minute capacity to bind to microsomal proteins

covalently, activation by microsomes in the presence of NADPH stimulated binding. Studies with L5178Y lymphoblasts have shown that very small amounts of PMM were detectable within 1 min in cells treated with HMM.[104] The available evidence does not yet allow judgement to be made as to the relative importance of hepatic and extrahepatic metabolism for antitumour activity.[106]

Two obvious candidates for the role of active metabolites are the carbinolamine and formaldehyde. Rutty et al. have used semicarbazide in an attempt to differentiate between the cytotoxicity of the carbinolamine per se and the formaldehyde it releases.[100] Semicarbazide (38) is claimed to react with formaldehyde under the incubation conditions employed and thus act as a trapping agent. Pretreatment of PC6 cells with semicarbazide indeed protects against formaldehyde toxicity in vitro whereas the cytotoxicity of the carbinolamine was unaffected by its presence thus indicating that the carbinolamine apparently does not work through the release of formaldehyde.

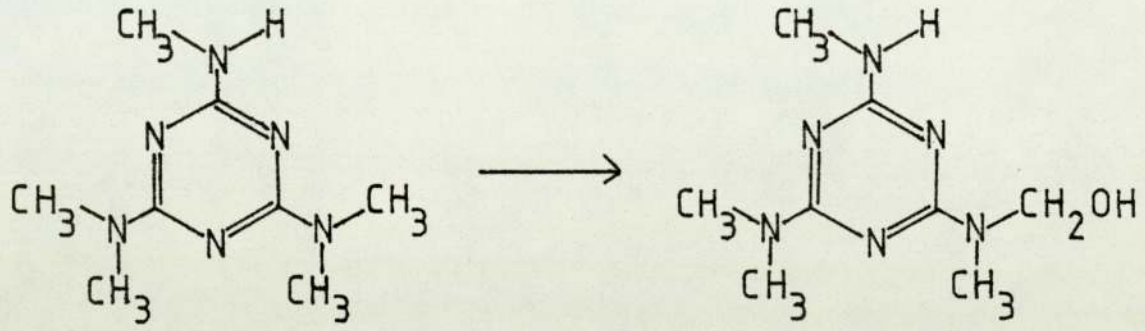


(38)

The time course chosen for the incubation was 72h whereas the half-life of the carbinolamine with respect to its breakdown to PMM and formaldehyde is less than 1h. Thus for a major period of the incubation time, PMM was presumably present rather than the carbinolamine.

There are indications that PC6 cells are capable of metabolising HMM[105] and thus it is possible that

carbinolamines, e.g. (39), are being continuously generated from PMM within the system (scheme 1.6).



(33) (39)

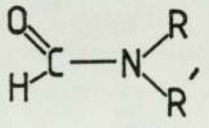
Scheme 1.6. Production of carbinolamines from PMM

Cell lines which are insensitive to HMM *in vivo* e.g. the TLX5 and L1210 tumours have been shown to be protected by semicarbazide from the cytotoxic effects of the carbinolamine *in vitro* and it has been suggested that formaldehyde alone accounts for the non-selective cytotoxicity in these cases. Contradictory evidence has however been obtained by Ross *et al.*[107] for the L1210 tumour where semicarbazide failed to protect against the cytotoxic effects of the carbinolamine. Differences in cell lines of the L1210 was suggested as a possible reason for this discrepancy. In addition the Walker carcinoma, which is a tumour sensitive *in vivo* to HMM, is protected from the cytotoxicity of the carbinolamine by semicarbazide.[100] The significance of these semicarbazide

protection experiments is therefore difficult to interpret and the role of the carbinolamine relative to that of formaldehyde for antitumour activity remains unclear.

1.5 N-Methylformamide

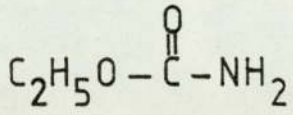
In 1953, whilst evaluating a series of formamides as possible solvents for the parenteral administration of novel compounds in an antitumour screening programme, Clarke *et al.* discovered the antitumour properties of N-methylformamide (NMF; 13) and formamide (F; 40)[108]. Within the same report it was stated that preliminary observations with more than 60 formamides had failed to uncover any other with significant activity against the murine Sarcoma 180. Two years later, Furst *et al.*[109] in a study of 83 formamides and related compounds tested against the Ehrlich ascites tumour reported that, again, only NMF and F demonstrated marked activity. However, N N-dimethylformamide (41), N N-diethylformamide (42) and N-ethylformamide (43) all appeared to possess marginal activity.



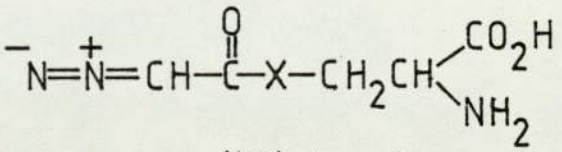
	R	R/
(40)	H	H
(13)	CH_3	H
(41)	CH_3	CH_3
(42)	C_2H_5	C_2H_5
(43)	C_2H_5	H

Early investigations of the mechanism of action of the compound revealed little save that it might interfere with

nucleic acid base synthesis.[110-114] Attempts were made to link the mechanism of action of NMF to those of urethane (44),[110-112] azaserine (45)[112,113,115] and DON (46).[115]



(44)



(45) X = O

(46) X = CH_2

Formate incorporation into the nucleic acids of the liver was shown to be stimulated by NMF[116,117] and it was thought that this might be involved in some way with the hepatotoxicity of the drug,[108,118] which has since proven to be the dose-limiting factor in its administration to patients.[118] This finding of hepatotoxicity in its first clinical trial in 1955[118] led to a loss of interest in its potential as a clinical agent; however clinical interest in the drug was recently revived with the discovery of its very marked activity against the N.C.I. panel of human tumour xenografts.[119] The compound is currently being reinvestigated in two phase 1 clinical trials in London and Amsterdam and others are about to be initiated in the U.S.A.

CHAPTER 2 Metabolism of the N-methylamino group

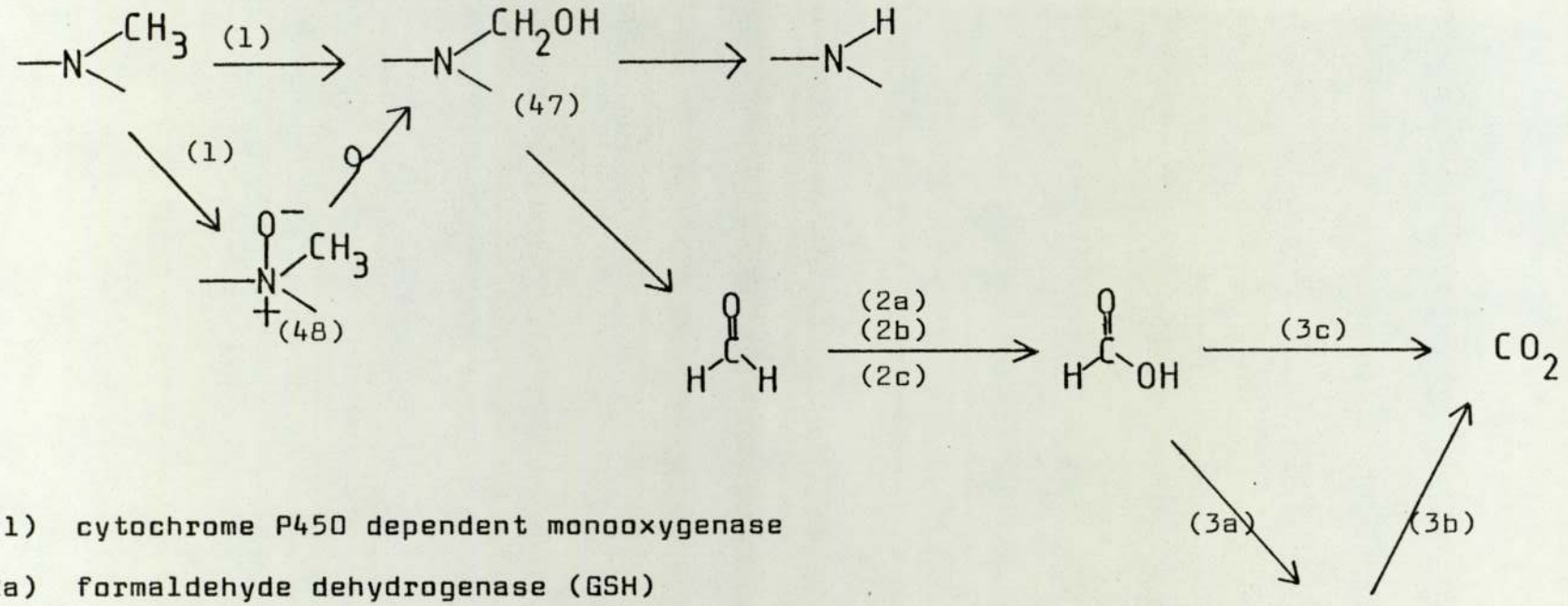
The major metabolic transformation for the N-methyl group involves N-demethylation to formaldehyde and the desmethylamine.[120,121] Two pathways are possible for this oxidation, either via direct C-oxidation to the carbinolamine (47) or via the N-oxide (48) and subsequent rearrangement to the carbinolamine (scheme 2.1). The formaldehyde generated by this pathway is then detoxified by oxidation to formate which in turn is oxidised to CO_2. This latter conversion can follow two pathways, either via a 10-formyltetrahydrofolate linked route or more directly through a peroxisomal catalase system.[121]

A metabolic pathway which reveals novel aspects of N-demethylation has recently been found for compounds such as N-methylbenzamide (49) (scheme 2.2).[122] The N-formyl metabolite (50) is evolved from the carbinolamine and is then metabolised to the desmethylamine without production of formaldehyde. The availability of this pathway to other N-methyl containing xenobiotics is unknown.

For all of the compounds, procarbazine, the dimethyltriazenes HMM and NMF, the following statements can be made:

i) Incubation of the drug with mouse or rat liver microsomes <u>in vitro</u> generates formaldehyde (exc. NMF).[18,123,51,52,98,119]

ii) <u>In vivo</u>, a formaldehyde precursor, presumably the carbinolamine or a carbinolamine conjugate is



(1) cytochrome P450 dependent monooxygenase

(2a) formaldehyde dehydrogenase (GSH)

(2b) aldehyde dehydrogenase

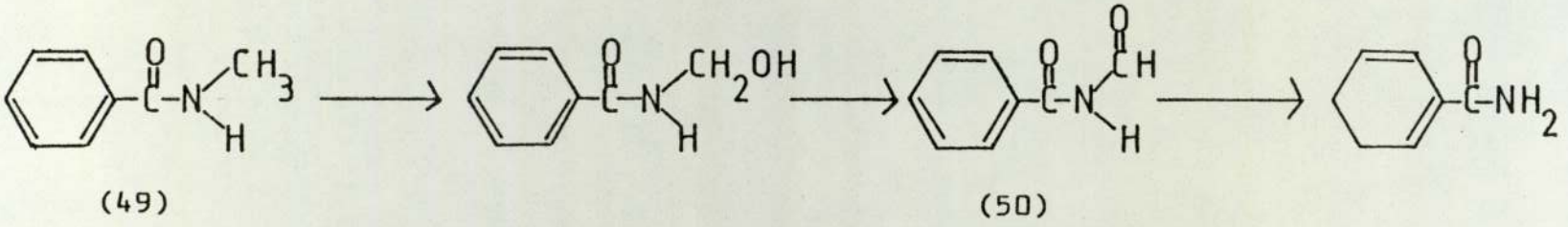
(2c) catalase

(3a) 10-formyltetrahydrofolate synthetase

(3b) 10-formyltetrahydrofolate dehydrogenase

(3c) catalase

Scheme 2.1 From N-demethylation to formaldehyde, formate and CO_2 production



(49) (50)

Scheme 2.2. Metabolism of N-methylbenzamide

metabolically produced after administration of the drug.[17,63,124,122]

iii) $^{14}CO_2$ is expired by mice or rats receiving the drug labelled with ^{14}C in its N-methyl position.[21,30-32,126,92,125]

There is little doubt, therefore, that all of the above compounds are oxidatively N-demethylated *in vivo* although for procarbazine this may not represent the major biotransformation (CO_2 may also derive from methanol evolved in procarbazine's metabolism, scheme 1.1). With the exception of NMF,[127] appreciable cytotoxicity *in vitro* of the above compounds is achieved only after microsomal activation.[36,52,2,98] NMF is relatively non-toxic *in vitro* under all conditions investigated to date.

The conjecture is that metabolism is also required *in vivo* for antitumour activity. This is a hypothesis that is very difficult to prove. Mention has already been made that it is insufficient for a species to simply be cytotoxic; it must also possess the necessary capabilities to be present at a tumour target in sufficiently high concentration to exert that cytotoxicity.

The carbinolamines of NMF,[127] the dimethyltriazenes,[2] and HMM[100] are all markedly more cytotoxic *in vitro* than their respective N-methyl analogues. This fact has implicated them as being possible 'active metabolites' *in vivo*. When these compounds

are administered *in vivo*[98,2] they demonstrate similar toxicity and antitumour activity to their N-methyl precursors except for NMF whose carbinolamine is very much less toxic and possesses reduced antitumour activity.[127] This does not necessarily invalidate the carbinolamine as a potential 'active metabolite' since its generation *in vivo* from a precursor may produce more effective levels at a tumour site than after injection of the carbinolamine as such. This might be the case where prolonged levels of a metabolite are required rather than just a very high initial peak level.

The available evidence does not yet allow judgement to be made as to the relative importance of the carbinolamine and formaldehyde for antitumour activity. Gescher *et al.*[120] have suggested that the carbinolamine may represent a nascent form of formaldehyde which escapes the detoxification mechanisms (scheme 2.1) of the liver. If this is so then the stability of the carbinolamines may well be important. An unstable carbinolamine (e.g. from aminopyrine[120]) will rapidly decompose to release its formaldehyde and may therefore not reach a potential target. On the other hand, too stable a carbinolamine may decompose at too slow a rate within a tumour to produce useful cytotoxicity. Thus a balance may be required possibly explaining why only certain N-methyl containing xenobiotics are antitumour agents. If this is the case it may be possible to 'tune' the stability of the carbinolamines to achieve the desired therapeutic objective.

Although the carbinolamine and formaldehyde may well represent the most cytotoxic species generated by NMF and HMM (and this is not yet certain for NMF), both procarbazine and the dimethyltriazenes generate additional highly cytotoxic species. These may play an important if not key role in their mechanisms of action as antitumour agents. If this is so then it is unclear why the N-methyl group should be a requirement for antitumour activity within these molecules if this metabolic pathway is not important.

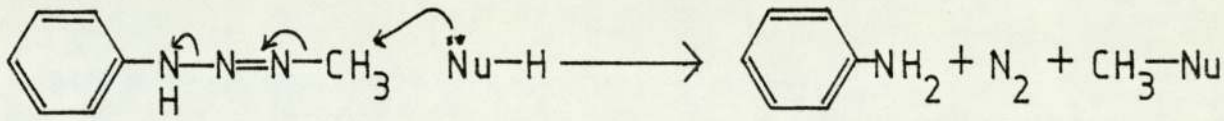
CHAPTER 3 The role of alkylation in the mode of action of N-methyl containing antitumour agents

3.1 Introduction

Since HMM, procarbazine and the dimethyltriazenes all produce electrophilic species along their metabolic pathways it is reasonable to suspect that 'alkylating activity' may be responsible for their antitumour activity. The nature of the alkylation for all of these compounds is unclear but the possibilities include hydroxymethylation (via formaldehyde), aminomethylation (via a carbinolamine) and methylation (via an S_N2 process). Similarly the target of alkylation crucial to the antitumour process remains unidentified. The ability of these molecules to alkylate and cross-link biomolecules is reviewed in the following pages.

3.2. Triazenes

The dialkyltriazenes are known to be potent carcinogens.[128] Whereas 1-phenyl-3,3-dimethyltriazene (27; R= CH_3) acts as a systemic carcinogen, 1-phenyl-3-monomethyltriazene (29; R=H) produces tumours at the injection site implicating it as the proximate carcinogen evolved from the dimethyl compound.[129] Further the monomethyltriazene is a potent alkylating agent and methylates biomolecules, probably through an S_N2 process (scheme 3.1).[130]



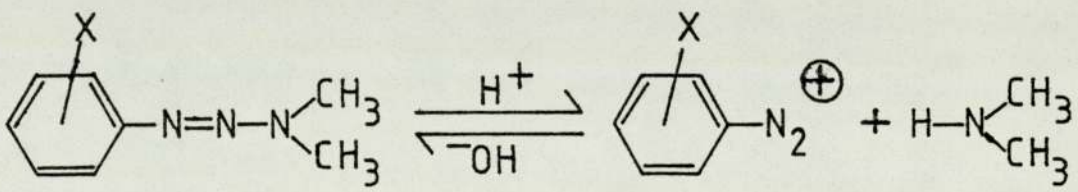
Scheme 3.1

Alkylation both *in vivo*[129] and *in vitro*[131] has been demonstrated by the production of 7-methylguanine in the DNA and RNA of rat liver cells. These alkylating and carcinogenic abilities are not properties exclusive to the N-methyl containing triazenes as the monoethyltriazenes behave in an analogous manner *in vitro* producing 7-ethylguanine in the DNA and RNA of these tissues.[131] Although 7-methylguanine was detected in the DNA of livers of rats after administration of 1-phenyl-3,3-dimethyltriazene no tumours were observed in this organ although they appeared in other tissues.[129] Thus the link (if any) between alkylation, as determined by methylation of the N(7) position of guanine, and carcinogenesis is a tenuous one.

The possibility that the dimethyltriazenes function as anti-tumour agents in a manner similar to the 'classical' alkylating agents appears unlikely. Several pieces of evidence militate against this. Firstly Audette *et al.*[51] have demonstrated that the triazenes are active against the R1 and TLX5 lymphomas which are claimed to be insensitive to difunctional and monofunctional alkylating agents. Furthermore they are

active against the PC6A plasmacytoma, a tumour claimed to be insensitive towards monofunctional alkylating agents.[51] Although very great assumptions are being made here about the biological nature of these tumours, the observation that nitrogen mustard (3) and cyclophosphamide (5) retain full activity against an L1210 cell line made resistant to DTIC (11) substantiates the assertion that a different mechanism of action is involved.[54]

The involvement of methylation in the antitumour activity appears unlikely in the light of the fact that human malignant melanoma cells which are either sensitive or resistant to DTIC are methylated by the drug to an equal extent *in vitro*.[132] In addition di- and monoethyltriazenes are generally inactive as antitumour agents,[2] although monoethyltriazenes alkylate RNA and DNA as efficiently as monomethyltriazenes.[131] The diazonium ion (51) is another electrophilic species generated by the dimethyltriazenes and



(51)

Scheme 3.2 Protolysis of dimethyltriazenes

results from heterolytic fission of the molecule (scheme 3.2) in protic media.[52] Audette *et al.* have shown these species

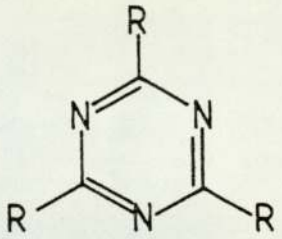
to be extremely cytotoxic towards TLX5 lymphoma cells *in vitro*.[51] Connors *et al.* have synthesized a series of dimethyltriazenes which protolyse over a large range of different rates.[52] The rate of protolysis was shown not to correlate with the antitumour activity *in vivo* suggesting that production of the diazonium ion is unrelated to the antitumour process.

The involvement and the importance of the carbinolamine and the formaldehyde it generates are unknown at present.

Therefore it appears that although the dimethyltriazenes generate a variety of electrophilic species, none has yet been positively linked with antitumour activity. It is feasible that no single species is responsible for activity but instead a cascade of reactive moieties produces the antitumour response. As Stevens has suggested, patients receiving DTIC may well in fact be receiving combination chemotherapy.[130]

3.3. Hexamethylmelamine (HMM)

HMM (12) and triethylenemelamine (7) are both active against the Walker 256 carcinoma.[133] The similarities between the two compounds have often been referred to and this originated from the investigations of Hendry *et al.* in the early 1950's [133-135].



R

(12) $-N(CH_3)_2$

(7) $-N\langle CH_2-CH_2\rangle$

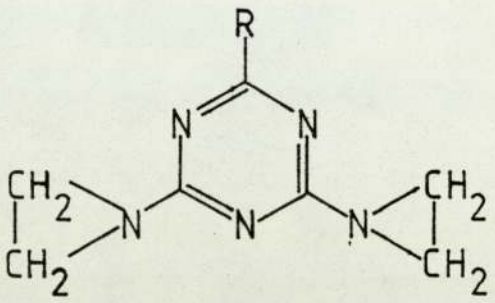
(52) $-N(CH_2OH)H$

Hendry et al. in their studies on the antitumour mechanism of urethane (44) examined compounds which, like urethane, could condense with amino groups.[133-135] They screened a series of compounds which were employed as industrial cross-linking agents in the fabric industry. Since these agents could modify the properties of certain fabrics by covalently linking peptide chains of textile fabrics it was argued that they might also cross-link biologically essential molecules such as DNA in the manner of the 'mustard' group. Out of these compounds emerged triethylenemelamine (7)[133] and trimethylolmelamine (52)[135] as active against the Walker 256 carcinoma. The methylolamine group is considered to react towards nucleophiles in a manner similar to the aziridinyl functionality (reactions a and b).

a) $Nu-H + \langle CH_2-CH_2\rangle N-X \longrightarrow Nu-CH_2CH_2NH-X$

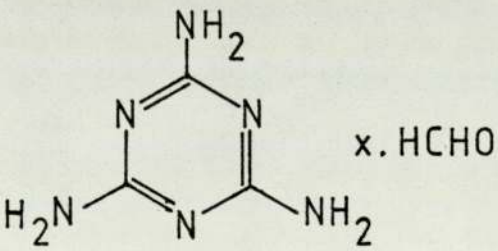
b) $Nu-H + HO-CH_2NH-X \longrightarrow Nu-CH_2NH-X + H_2O$

Structural requirements for molecules containing aziridinyl functionalities attached to an s-triazine ring were defined.[133] In general at least two aziridinyl groups were necessary for activity (53), although a few compounds containing only one aziridinyl group showed a very small degree of activity. Many modifications were permitted for R with activity being retained. The general requirement for two aziridinyl groups suggested that cross-linking might be responsible for the antitumour activity of these compounds.



(53)

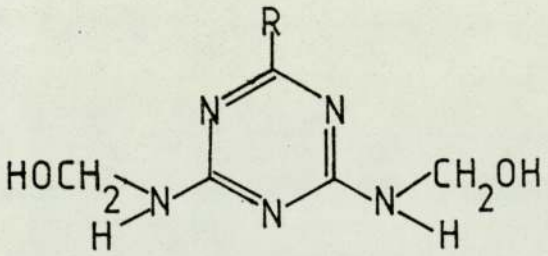
Condensates of melamine and formaldehyde were tested against the Walker 256 carcinoma and the results obtained are shown in table 3.1.[134]



x	optimal inhibition of tumour growth %
0	22
1	42
2	84
3	100
4	97
5	74
6	61

Table 3.1 Inhibition of the Walker 256 carcinoma by condensates of melamine and formaldehyde.

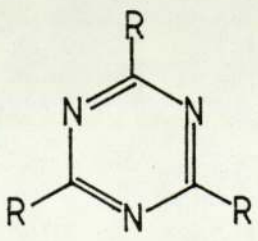
The condensate containing three equivalents of formaldehyde to one of melamine produced the best inhibitions against this tumour. These compounds are difficult to characterise and obtain pure and it is probable that mixtures are represented, however the 3:1 condensate was probably essentially trimethylolmelamine (52). When compounds containing two hydroxymethylamino groups attached to an s-triazine (54) were investigated, they were often devoid of antitumour activity suggesting that the group R in (54) is playing an important role in the antitumour requirements of the molecule.[134]



(54)

Thus the suggestion is that polymethylols may well act as biological cross-linking agents. No evidence has yet been presented that HMM is capable of producing polymethylols but it is a possibility. The stepwise pathway through which HMM is metabolized (scheme 1.4) suggests production of single methylols and subsequent breakdown to the desmethylmelamine before further activation; however polymethylols may still be produced in lesser quantities.

The polymethylols, trimethyltrimethylolmelamine (55) and hexamethylolmelamine (56) have been synthesized and tested



(55) R = $-N(CH_2OH)(CH_3)$

(56) R = $-N(CH_2OH)(CH_2OH)$

against the PC6A plasmacytoma both *in vitro*[100] and *in vivo*.[100] *In vivo* both are active although no more so than HMPMM (the monomethylol; 37) or HMM (12) itself[98] and *in vitro* both demonstrate activity similar to that of the monomethylol.[100] They are however chemically unstable and decompose to release formaldehyde which may be responsible for the observed cytotoxicity.[100]

DNA and DNA-protein cross-linking in L1210 cells after exposure to trimethyltrimethylolmelamine has recently been investigated by Ross *et al.*[107]. The dose of the drug required to elicit cytotoxicity was first established and at this dose substantial cross-linking of both of the above types was found. However neither HMM activated with 9,000 x g liver supernatant nor HMPMM produced cross-links at their respective cytotoxic doses. Formaldehyde even at non-toxic levels produced extensive DNA-protein cross-linking but no inter-strand links. The conclusion was that cross-linking was not of primary importance for the drug induced cytotoxicity of HMM in these cells. In contrast, Muindi *et al.* found that HMPMM was able to induce DNA-protein links in L1210 cells but demonstrated that these were reversible by preincubation with semicarbazide implicating formaldehyde as responsible.[136] With the same compound neither DNA nor DNA-protein links were found in PC6 cells after exposure to cytotoxic levels, although this tumour is sensitive to the drug *in vivo*. They concluded that the cytotoxicity of HMPMM was not attrituble to DNA cross-linking.

Morimoto *et al.* using Lieberman plasma cells, a tumour sensitive to HMM *in vivo*, have shown that both DNA and RNA synthesis are dramatically inhibited by the drug.[137] Radiolabelled HMM (or more likely a metabolite) was shown to be bound to both the DNA and RNA within these cells. DNA and RNA inhibition have also been demonstrated in Ehrlich ascites cells.[138]

The role of cross-linking, therefore, appears from the above to not play a substantial part in the antitumour activity of HMM although DNA and RNA syntheses are inhibited.

It is of mechanistic chemical interest that whereas neither HMM nor its metabolites appear to react with nitrobenzylpyridine,[92,100] a reaction considered a standard test of alkylating ability, the metabolites are clearly able to bind covalently to the macromolecules of cells.[103]

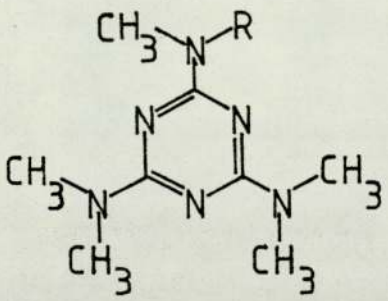
Formaldehyde is known to be a weak alkylator[139] and mutagen.[140] It is able to bind to DNA,[140] RNA[141] and proteins[142] and interfere with coupled phosphorylation.[143] Although studies have demonstrated that semicarbazide is able to protect against the cytotoxic effects of formaldehyde whereas HMM after microsomal activation often remains as toxic in the presence or absence of semicarbazide the role of formaldehyde cannot be dismissed.[100] It is feasible that formaldehyde may only ever be released from the carbinolamine at the bionucleophilic target and thus may not have the opportunity to react with semicarbazide.

3.4 Procarbazine

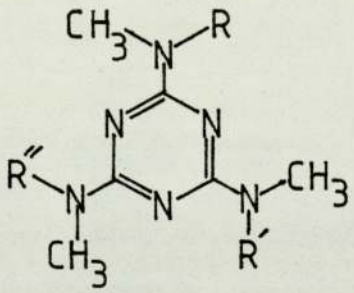
Procarbazine produces a multitude of potential alkylating species (scheme 1.1) yet virtually nothing is known of their role with respect to the antitumour action of the drug. Transmethylation of the intact N-methyl group of procarbazine onto the N(7) position of guanine in the cytoplasmic RNA of P815 leukaemia cells has been demonstrated by Kreis .[20] The extent of methylation however is unlikely to be lethal to the cells. Incorporation of precursors into the DNA, RNA and protein of L5178Y lymphoma cells is inhibited by the drug[144] and DNA fragmentation in various organs of Swiss mice has been demonstrated.[37] None of these effects have been shown to correlate with the antitumour activity of the drug.

CHAPTER 4 Aims of the present work

Cumber *et al.* in a search for analogues of HMM with enhanced water solubility synthesized and tested a series of substituted *s*-triazines against the ADJ/PC6A (PC6) murine plasmacytoma.[145] Their results indicate that *s*-triazines containing five exocyclic N-methyl groups (57) were, in general, active and those containing three (58) or four (59) N-methyl groups were sometimes active depending on the other exocyclic substituents.



(57)



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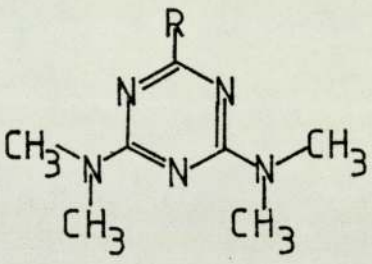
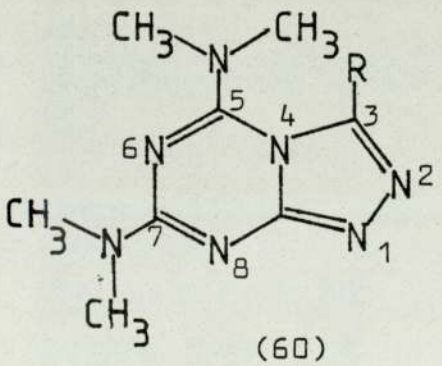


(59)

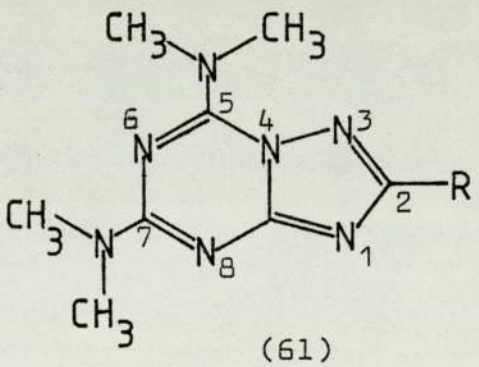
They concluded that for the series investigated there was no correlation between the rates of *in vitro* metabolism and the therapeutic indices achieved in the antitumour assays. Rutty *et al.* investigating a similar series of compounds came to the opposite conclusion; i.e. that a correlation did exist between these two parameters, but noted that there were some important exceptions.[98]

One objective, therefore, was to synthesize a series of simple substituted s-triazines containing from three to six N-methyl moieties in order to investigate their anti-tumour properties and metabolism both *in vivo* and *in vitro* in an attempt to determine the requirements for antitumour activity.

Considering the results for the PC6 tumour, a minimum requirement for activity within the methylmelamine structure appears to be about three or four N-methyl groups. A series of novel 3-substituted-s-triazolo [4,3-a] -s-triazines(60) and their isomers the 2-substituted-s-triazolo [2,3-a] -s-triazines(61) containing dimethylamino groups in their 5- and 7-positions were synthesized and their spectroscopic and biological properties investigated.



(60)



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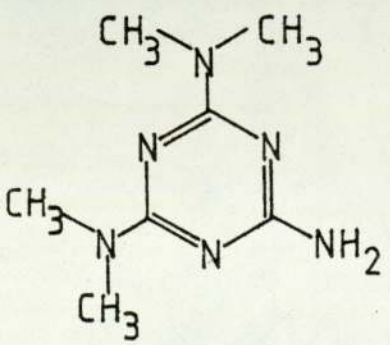


(62)

It was hoped that these bicyclic melamines might be similar enough to 2-amino-4,6-bis(dimethylamino)-*s*-triazine (62) (a compound known to be active against the PC6 tumour) that they could retain activity.

A test system was required to compare HMM and its analogues. The M5076 sarcoma was selected and its chemosensitivity to a wide variety of agents investigated.

Since formaldehyde has been implicated in the mechanism of action of HMM, its antitumour activity *in vivo* was also studied.

The final section of this thesis is concerned with N-methylformamide (NMF). The structural requirements necessary for the antitumour activity of the NMF molecule were examined. Since the drug was about to enter phase I clinical trials several aspects of its toxicology were studied and this led on to investigations of its potential use within combination chemotherapy.

SECTION 2

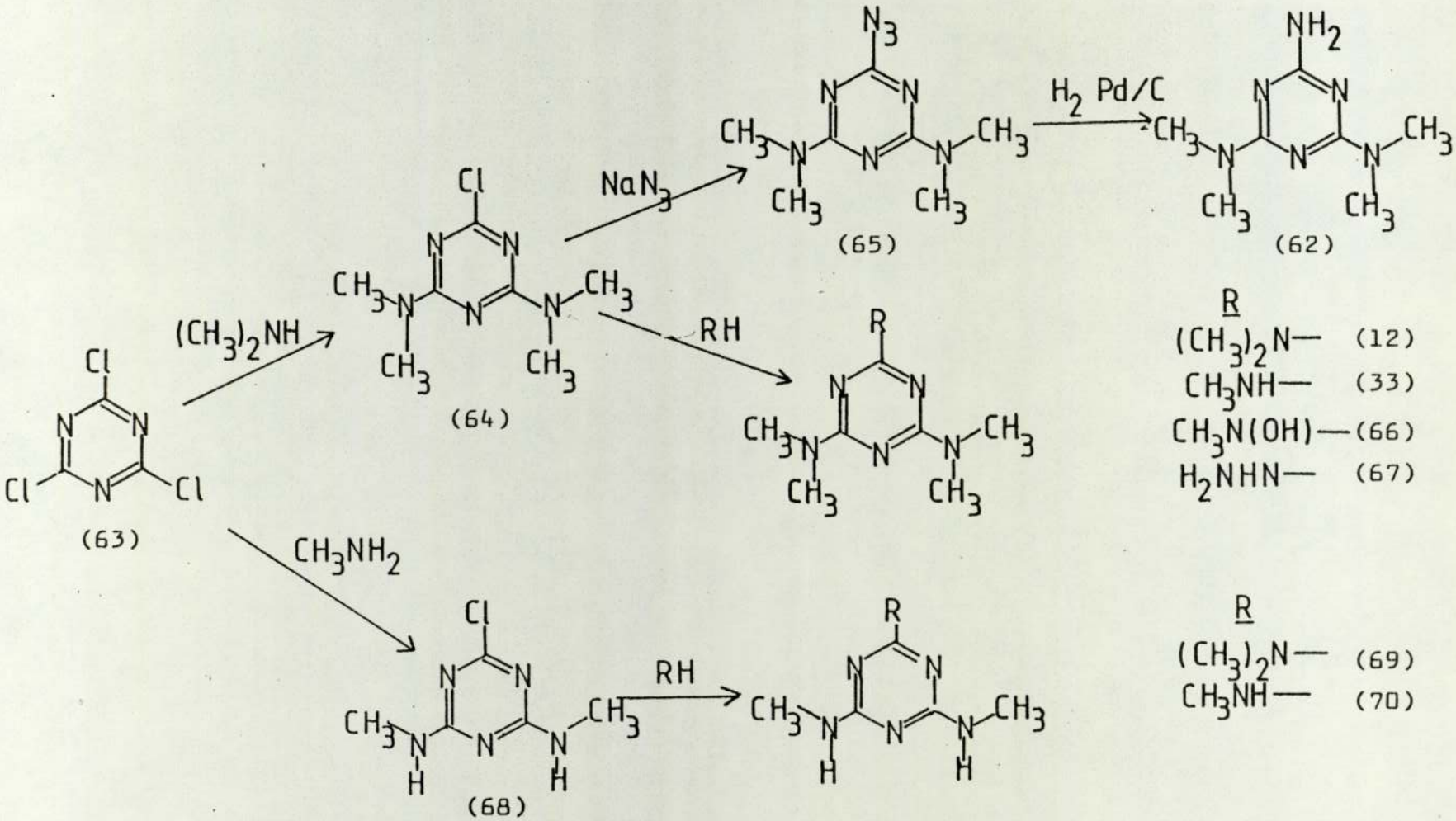
RESULTS AND DISCUSSION

Chapter 5 The synthesis of analogues of hexamethylmelamine (HMM)

5.1 Synthesis of 2,4,6-trisubstituted-s-triazines

The methods of Borkovec *et al.*[146], Thurston *et al.*[147] and Pearlman *et al.*[148] were used for these syntheses.

The synthesis of all the required 2,4,6-trisubstituted-s-triazines derived from the same starting material, cyanuric chloride (2,4,6-trichloro-s-triazine; 63). Stepwise substitution by nucleophiles of the chloro groups within this molecule is a relatively facile process. In preparing unsymmetrically substituted s-triazines the order of entry of the incoming nucleophile is most important.[148] Whereas the first two chloro groups can be substituted with ease by reasonable nucleophiles, high temperatures and/or pressure may be required to replace the third chloro group. It is therefore advantageous to insert the poorer nucleophile first if difficulty is encountered in introducing it at a later stage.[148] Alternatively it may be possible to insert a substituent which may later be modified to the desired moiety. These criteria formed the basis for the syntheses shown in scheme 5.1. Thus although 2-chloro-4,6-bis(dimethylamino)-s-triazine (64) was prepared at room temperature, heating under reflux was required for insertion of the third nucleophile. Azide ion, dimethylamine, methylamine, N-hydroxy-N-methylamine and hydrazine all displace the final chloro group easily under these conditions to yield compounds (65,12,33,66,67) however concentrated aqueous ammonia does not. It is possible to first insert the unsubstituted amino group at low temperature and then further insert two molecules of dimethylamine to afford 2-amino-4,6-bis(dimethylamino)-s-triazine (62), however a more recent



Scheme 5.1 Preparation of 2,4,6-trisubstituted-s-triazines

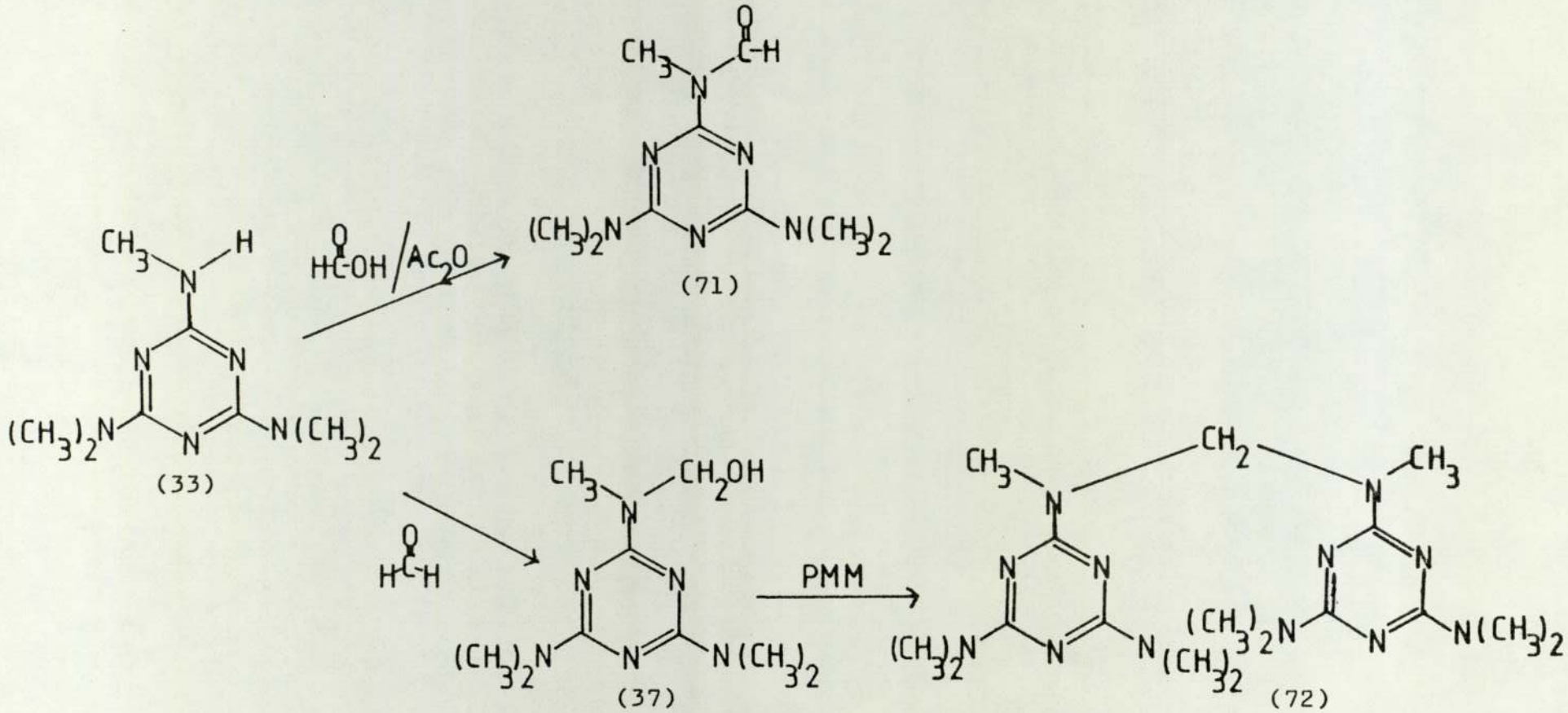
synthesis was available for this compound.[149] Reaction of 2-chloro-4,6-bis(dimethylamino)-s-triazine with sodium azide yielded the azide (65) and reduction of this with hydrogen over 5% palladium on charcoal afforded the amino-substituted product (62). 2,4,6-Tris(methylamino)-s-triazine (70) and 2-dimethylamino-4,6-bis(methylamino)-s-triazine (69) were both prepared from 2-chloro-4,6-bis(methylamino)-s-triazine (68) by substitution with methylamine and dimethylamine respectively. Either the free base or the amine hydrochloride plus sodium hydroxide could be used in these preparations.

Pentamethylmelamine (PMM, 2,4-bis(dimethylamino)-6-methylamino-s-triazine: 33) served as the source of several of the substituted pentamethylmelamine compounds (scheme 5.2). Reaction with formic acid and acetic anhydride yielded the N^2-formyl substituted compound (71)[150]. Reaction with formaldehyde afforded the carbinolamine (37) under mild conditions which reacted further with PMM when heated under reflux to produce $N^2,N^{2'}$-methylenebis(N^2,N^4,N^4,N^6,N^6-pentamethylmelamine; 72).

All structures were confirmed by n.m.r., mass spectral and infra red analyses and their purity confirmed by comparison of the m.p.s. with literature values.

5.2 Synthesis of 3-aryl-s-triazolo[4,3-a]-1,3,5-triazines

The known representatives of the s-triazolo[4,3-a]-1,3,5-triazine and s-triazolo[2,3-a]-1,3,5-triazine ring systems have been prepared either from s-triazoles[151] and aliphatic



Scheme 5.2 Preparation of N^2-substituted pentamethylmelamine compounds

precursors[152] or from the corresponding s-triazines. Three investigators have previously employed the latter strategy and all first prepared the appropriate 2-hydrazino-s-triazines (73).[153-155]

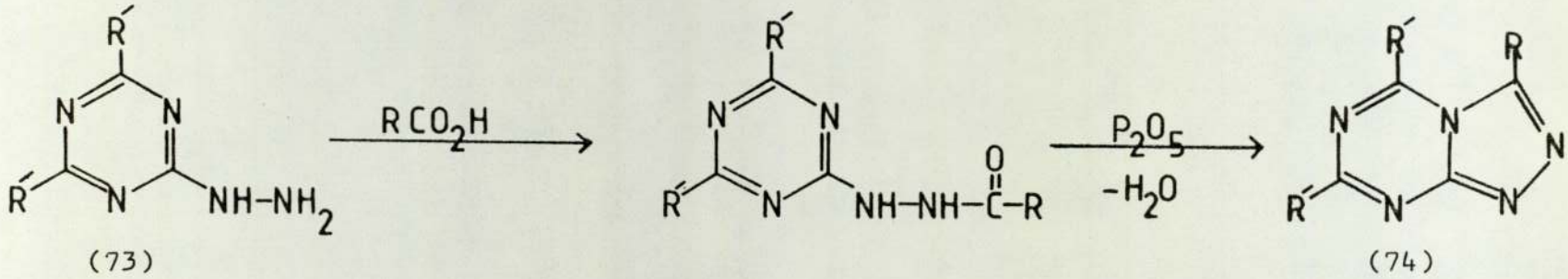
Desphande et al.[153], in his method, converted the hydrazino moiety to the hydrazide and then effected cyclodehydration of this compound to yield the s-triazolo[4,3-a]-1,3,5-triazine (74) (scheme 5.3).

De Milo et al.[154] simply condensed the 2-hydrazino-s-triazine with 1,1-thiocarbonyldiimidazole to yield the cyclic thione (75) (scheme 5.4).

The third approach, that of Kobe et al.[155], was employed for the present syntheses. The 2-substituted benzylidenehydrazino-4,6-bis(dimethylamino)-s-triazines (76) were first prepared by condensation of 2,4-bis(dimethylamino)-6-hydrazino-s-triazine (67) with the appropriately substituted benzaldehyde (scheme 5.5, table 5.1). The hydrazones (76) were then submitted to oxidative cyclization with lead tetraacetate to afford the 3-aryl-s-triazolo [4,3-a]-1,3,5-triazines (77) (scheme 5.5, table 5.1). The direction of ring closure is irrelevant within these compounds as the s-triazine ring is symmetrically substituted. The oxidation is effected at room temperature in either chloroform or dichloromethane and is complete within 2h. The characterization of these compounds is described in section 5.4.

5.3 Synthesis of novel 2-aryl-s-triazolo[2,3-a]-1,3,5-triazines

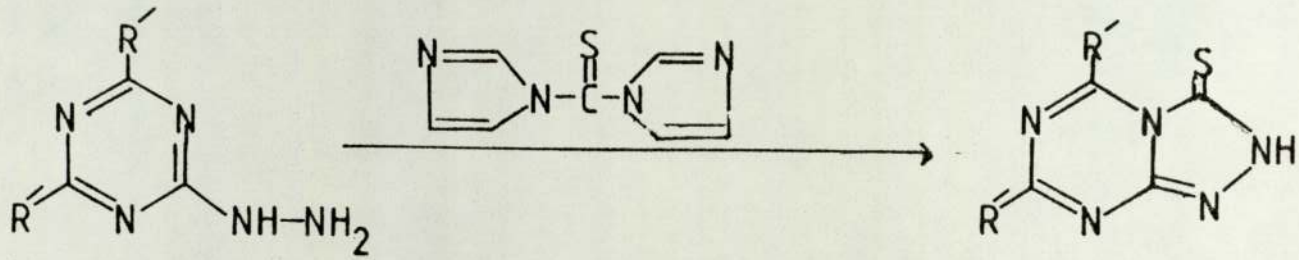
The 2-aryl-s-triazolo[2,3-a]-1,3,5-triazines (78) were prepared by a Dimroth-type rearrangement of the corresponding 3-aryl-s-



(73) (74)

Scheme 5.3 Synthesis of s-triazolo [4,3-a] -1,3,5-triazines

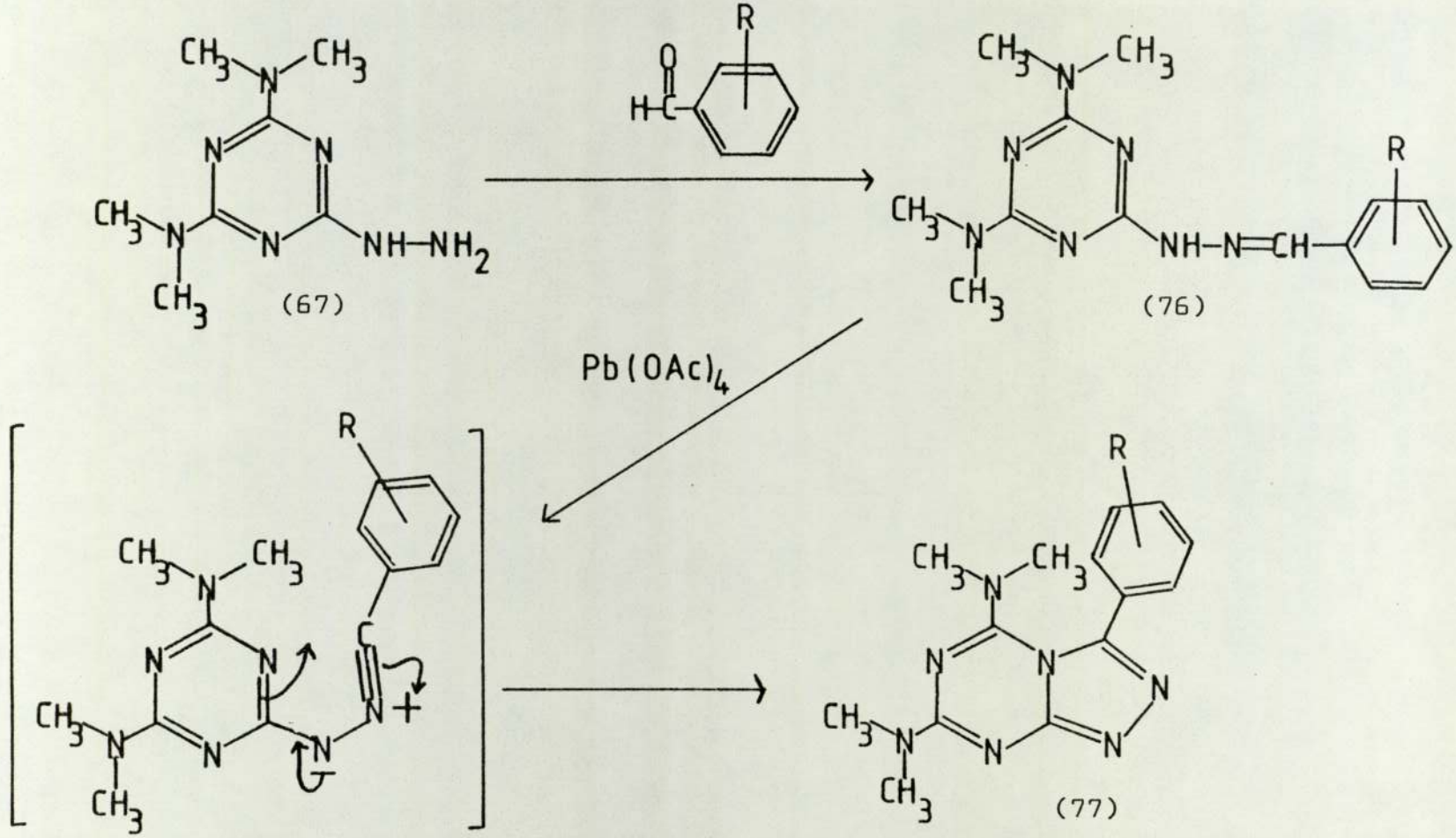
(method of Deshpande et al.[153])



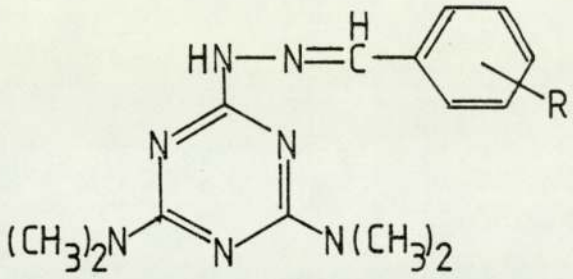
(75)

Scheme 5.4 Synthesis of s-triazolo[4,3-a] -1,3,5-triazines

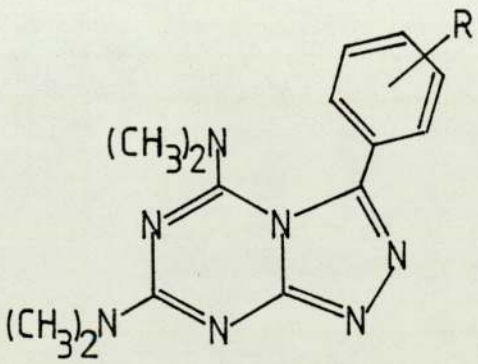
(method of De Milo et al.[154])



Scheme 5.5 Synthesis of the 3-aryl-s-triazolo[4,3-a]-1,3,5-triazines (method of Kobe et al.[155])



(76)



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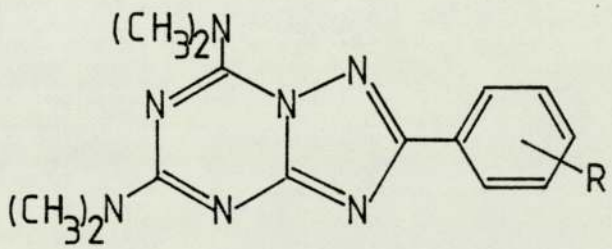


(78)

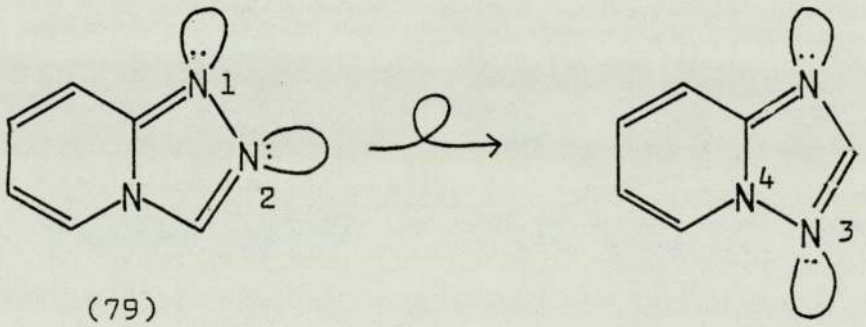
Compound number	R
76a	-H
76b	o - NO_2
76c	p - NO_2
76d	o - Cl
76e	m - Cl
76f	p - Cl
77a	-H
77b	o - NO_2

Compound number	R
77c	o - Cl
77d	m - Cl
77e	p - Cl
78a	-H
78b	o - NO_2
78c	o - Cl
78d	m - Cl
78e	p - Cl

Table 5.1

triazolo [4,3-a]-1,3,5-triazine (77) (scheme 5.6, table 5.1). This rearrangement is base-catalyzed and use of a 10% sodium hydroxide solution in either methanol or ethanol effects the rearrangement at room temperature, or more rapidly at elevated temperature.

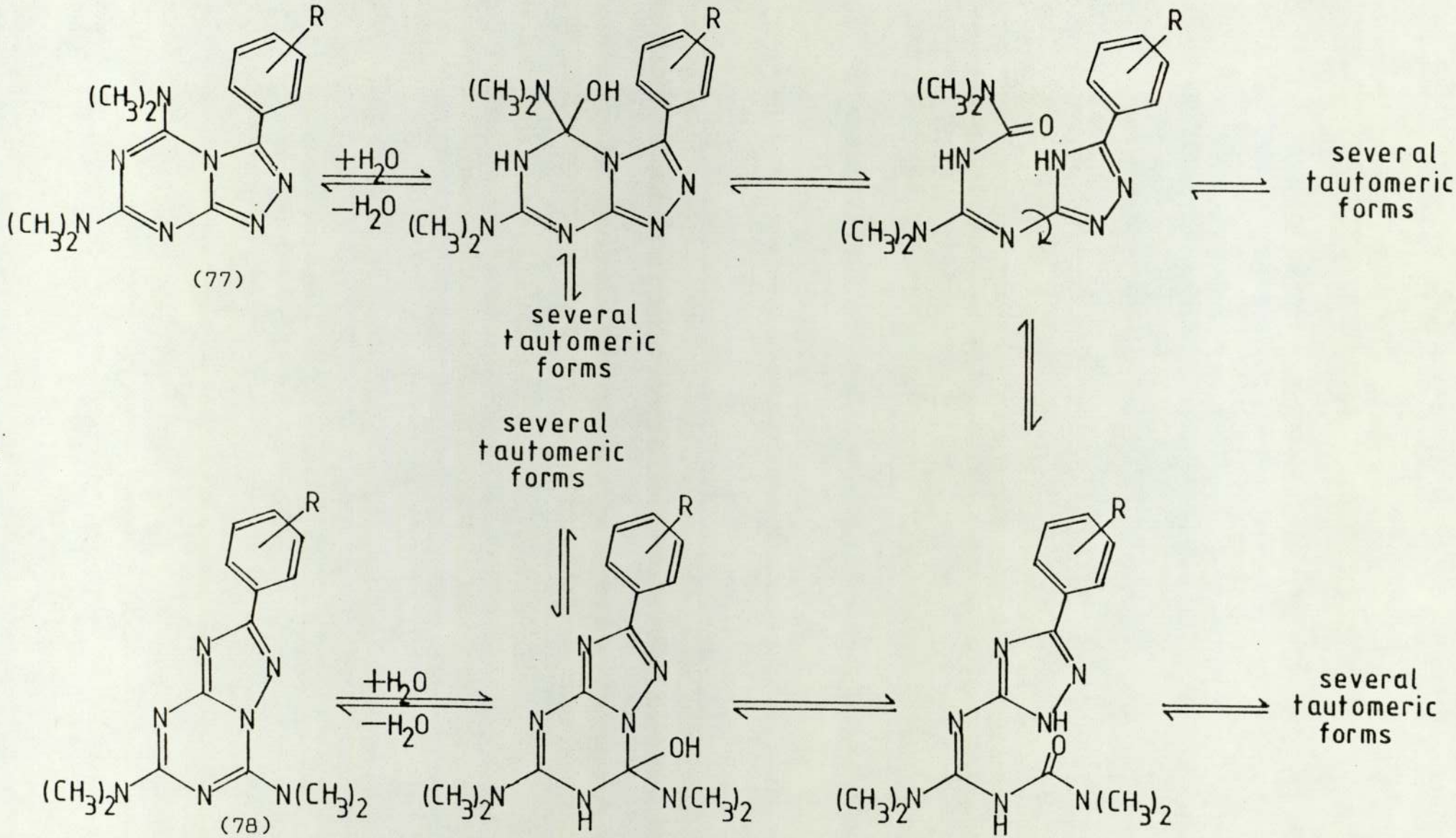
T.l.c. analysis (chloroform/methanol; 9/1) on silica gel plates of the reaction product revealed that the [2,3-a]-isomer was the faster moving species in each case and also that the reaction went to completion within 1h at elevated temperature. Guerret et al.[156] have suggested that the driving force for the rearrangement of the s-triazoloazines in general originates from the large interaction between the electron densities (Sp^2 orbitals) of N-1 and N-2 in the triazoloazine (79).



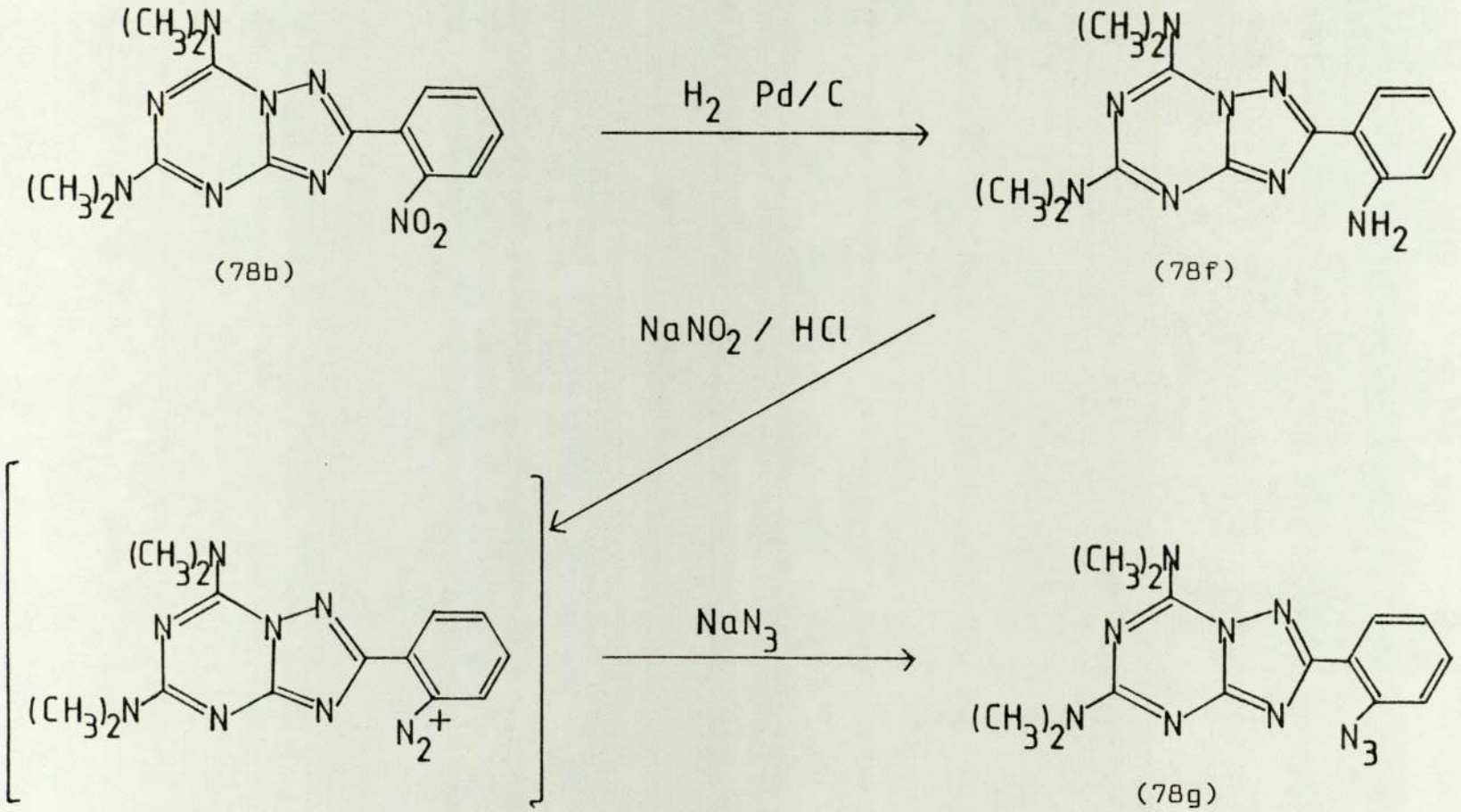
(79) (80)

This interaction can be relieved in the isomeric series (80) where the pair of electrons on N-4 contributes to the aromatic sextet of the triazole ring.

Two other analogues were also synthesized. The nitro group of (78b) was reduced to an amine (78f) function by hydrogen over 5% palladium on charcoal. Diazoatisation of the amine followed by reaction with sodium azide yielded the azide (78g) (scheme 5.7).



Scheme 5.6 Dimroth-type rearrangements of the 3-aryl-s-triazolo[4,3-a]-1,3,5-triazines (77)

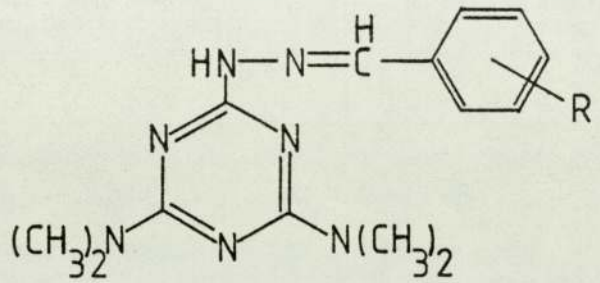


(78b) (78f) (78g)

Scheme 5.7

5.4. Spectral characteristics of the 2-substituted benzylidenehydrazino-4,6-bis(dimethylamino)-s-triazines

The ^1H n.m.r spectra of the 2-substituted benzylidenehydrazino-4,6-bis(dimethylamino)-s-triazines (76) indicate the N-methyl singlet to resonate between 3·11 and 3·20 δ (table 5.2).



(76)

R	Compound number	N-CH_3	-N=CH-Ar
-H	76a	3.12	9.41
o-NO_2	b	3.20	8.37
o-Cl	d	3.17	9.20
m-Cl	e	3.17	8.83
p-Cl	f	3.11	8.63

Table 5.2. Chemical shift values (δ) of the protons in the 2-substituted benzylidenehydrazino-4,6-bis(dimethylamino)-s-triazines

The mass spectra of these compounds are as indicated in appendix 5.1. The pattern of breakdown for the phenyl (76a) and chlorophenyl (76 d - f) substituted analogues was quite distinctive (fig. 5.1). Losses of imine molecules and methyl

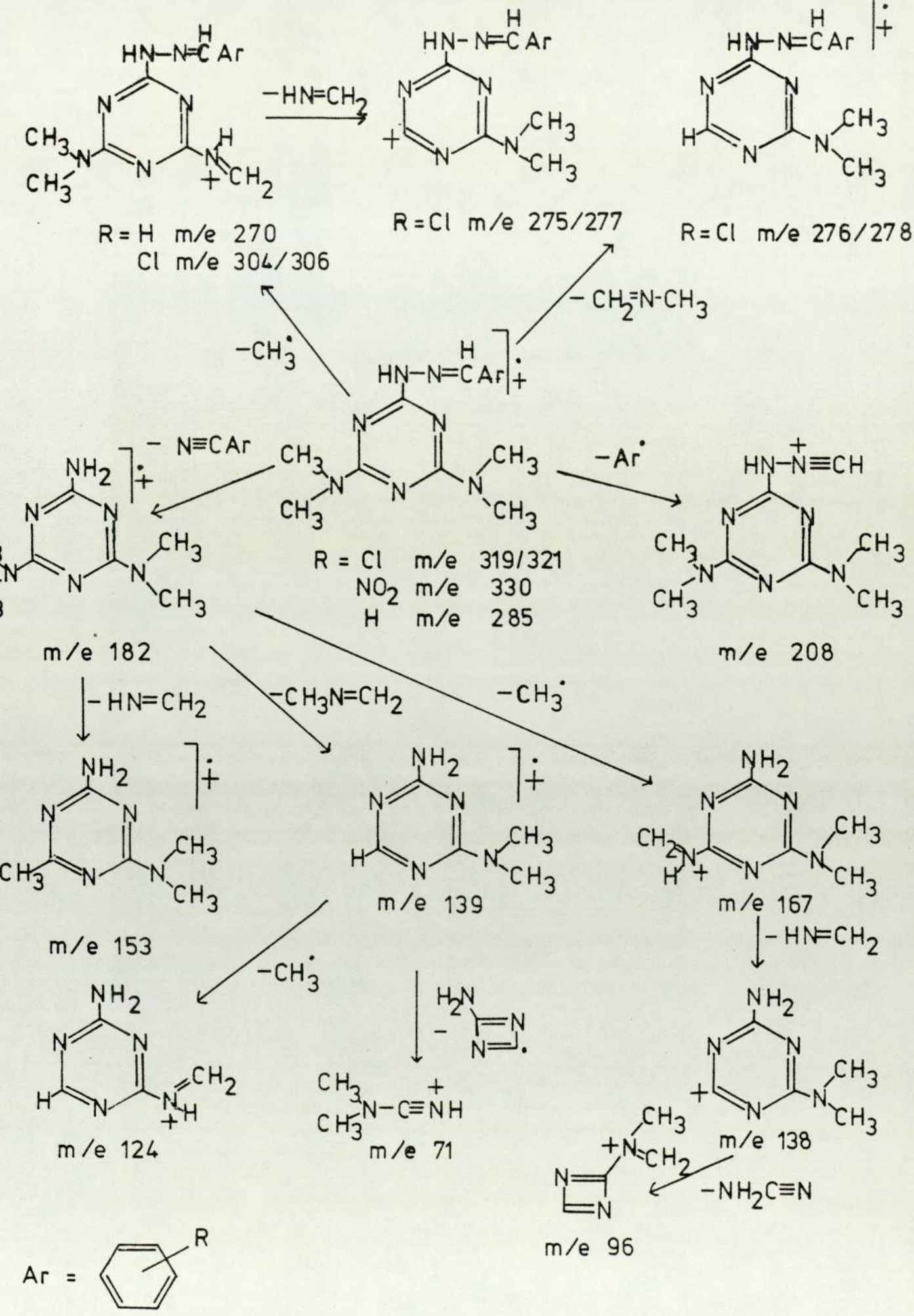


Fig 5.1 Possible mass spectral fragmentation pathways of the 2-substituted benzylidenehydrazino-4,6-bis(dimethylamino)-s-triazines.

radicals from the intact molecule occur to a minor degree. The major fragmentation produces the $^{m}/e$ 182 peak and subsequently demonstrates losses characteristic of the methylmelamines. This is the case also for the nitro analogues (76b,c).

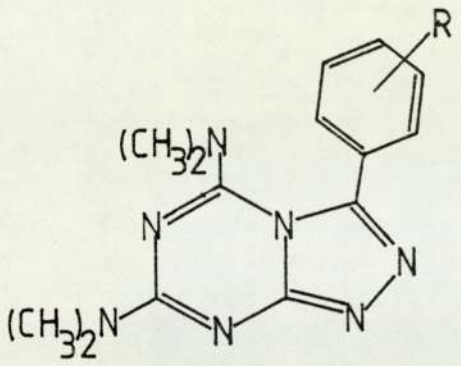
Two important absorbances are demonstrated in the U.V.spectra of these compounds; a major peak at 227-229 nm and a minor peak occurring at 305-317 nm. Addition of acid increases the intensity of the minor peak relative to the major with a concurrent hypsochromic shift (3-10 nm). Addition of base has no effect.

A peak at 810-820 cm^{-1} is present within the i.r. spectra of all of these compounds. This band has been assigned to an out-of-plane motion of the triazine ring and is present within all triazine molecules.

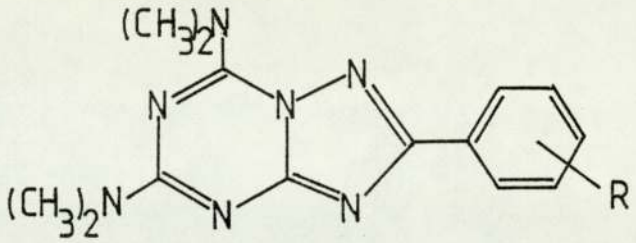
5.5 Spectral characteristics of the 3-aryl-s-triazolo-[4,3-a]-1,3,5-triazines and the 2-aryl-s-triazolo[2,3-a]-1,3,5-triazines

The n.m.r. and u.v. spectra are characteristic for each isomer within these two series.

Structures (77) and (78) may easily be differentiated by the chemical shift positions of their N-methyl groups (table 5.3). The 7-$N(CH_3)$ resonance is in approximately the same position in both isomers whereas the 5-$N(CH_3)$ singlet has shifted significantly in structure (77) as compared to (78). This shift



(77)



(78)

Compound number	R	5 - N($\underline{CH_3}$)	7 - N($\underline{CH_3}$)
77a	-H	2.67	3.23
77b	$\underline{o}$ - NO_2	2.55	3.27
77c	$\underline{o}$ - Cl	2.60	3.23
77d	$\underline{m}$ - Cl	2.73	3.23
77e	$\underline{p}$ - Cl	2.73	3.25
78a	-H	3.53	3.19
78b	$\underline{o}$ - NO_2	3.47	3.20
78c	$\underline{o}$ - Cl	3.55	3.20
78d	$\underline{m}$ - Cl	3.48	3.17
78e	$\underline{p}$ - Cl	3.53	3.20
78f	$\underline{o}$ - NH_2	3.43	3.17
78g	$\underline{o}$ - N_3	3.53	3.20

Table 5.3 Chemical shift values (δ) of the protons in the [4,3-a] and [2,3-a] series.

may be attributed to the peri-type interaction between the two substituents at the 3- and 5-positions in (77) resulting in the 5-N-methyl groups moving out of the plane of the triazine ring. Since there is no substituent at 3- in (78), no such steric interaction is anticipated.

In the same manner as the 2-substituted benzylidenehydrazino-4,6-bis(dimethylamino)-s-triazines the [4,3-a]-series demonstrate two maxima in their u.v. spectra. Again the major peak is in the region 228 - 238 nm and the minor peak in the region 260 - 275 nm. Addition of acid produces a hyperchromic effect in the minor peak such that it increases in absorbance above the other peak. It also demonstrates a small hypsochromic effect (1 - 10 nm).

The [2,3-a] - series possess only one major peak (227 - 244 nm) in their u.v. spectra with a second peak (253 - 335 nm) which is generally only manifested as a shoulder on the first or if shifted significantly is seen as a very minor peak. Addition of acid produces no change in absorbance and any change in the λmax is negligible. Addition of base to either s-triazolo-1,3,5-triazine has no effect on the u.v. spectra.

The mass spectra of both isomers (77) and (78) show molecular ion peaks in high abundance. Losses from the dimethylamino groups of the s-triazine ring represent a major feature in the spectra of both isomers. Production of the peaks $^{m}/e$ 137,96, 82,71,69 and 67 are common to almost all the analogues. These

probably represent fragments derived from the s-triazine component of the molecule (fig. 5.2).

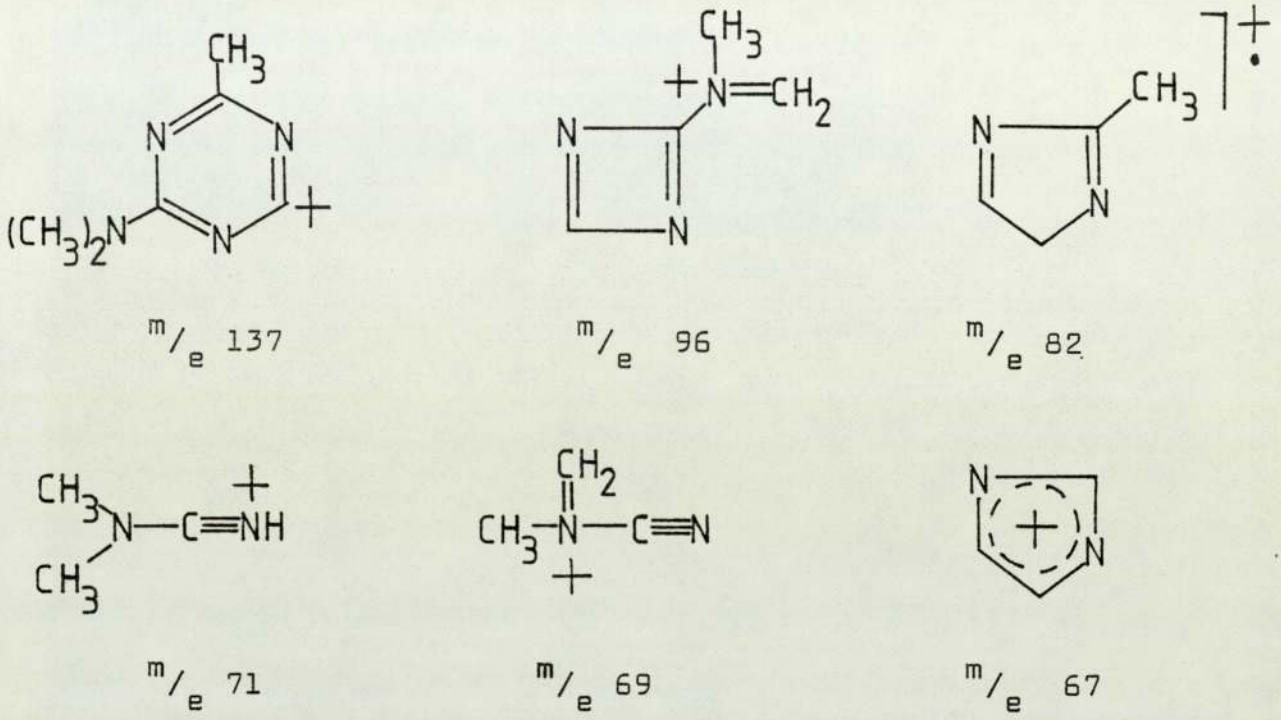


Fig. 5.2 Probable mass spectral fragments derived from the aryl-5,7-bis(dimethylamino)-s-triazolo-1,3,5-triazines

Chapter 6 The M5076 (M5) reticulum cell sarcoma

6.1 Introduction

Recently a new murine tumour, the M5076 (M5, M5076/73A) reticulum cell sarcoma has been introduced for experimental use[157,158]. The tumour arose as a spontaneous neoplasm in the ovary of a C57/BL mouse.[159] Initially characterized as an ovarian carcinoma, histological and immunological studies now indicate it to be macrophagic in origin.[158] Studies have demonstrated that its metastatic spread is highly unusual with metastases appearing primarily in the visceral organs, lung lesions being rare.[159,160] This property of the M5 renders it an attractive model for the study of tumour spread and colonisation.

HMM was reported active against this tumour,[157] and so the system was developed further for screening purposes and antitumour assays were conducted against it.

6.2 Establishment of the test system

In setting up a tumour as a screening model, several parameters have to be established. The first is the strain of mouse to be used as host. The tumour arose in a C57/BL mouse and these have generally been employed, however BDF_1 mice (hybrids of the C57/BL and the DBA/2 mice) were more readily available and are accepted as a possible alternative by the N.C.I.[161] These were used and no problems were encountered.

The tumour was originally considered to be an ovarian carcinoma and so female mice have always been employed as hosts - this practice was continued in these investigations. At least two sites of tumour implantation were feasible. Whereas American workers favour subcutaneous implantation in the flank, Italian workers prefer intramuscular implantation in the leg. The advantage of the leg muscle is that the shapes of large tumours at this site tend to be more reproducible, being enclosed fairly tightly by the skin of the leg; furthermore the tumour is more accessible to measurement by calipers. Tumour suspensions were favoured over fragments for the following two reasons. Firstly it is possible to count the number of cells in a suspension enabling greater reproducibility in inoculating mice with the same quantity of cells. Secondly evidence has been reported showing that the use of fragments can lead to selection of particular cell lines with a consequent loss of tumour heterogeneity.[162]

Three possible end points were available for the evaluation of antitumour activity; increase in survival time of treated mice over controls, tumour weight inhibition and tumour volume inhibition. The accumulated death days of 212 mice injected intramuscularly with 10^6 cells are illustrated in fig. 6.1. These represent the control mice of 7 experiments and the range of medians lies between 31 and 39 days (table 6.1), no survivors being obtained. The median rather than the mean is preferred.

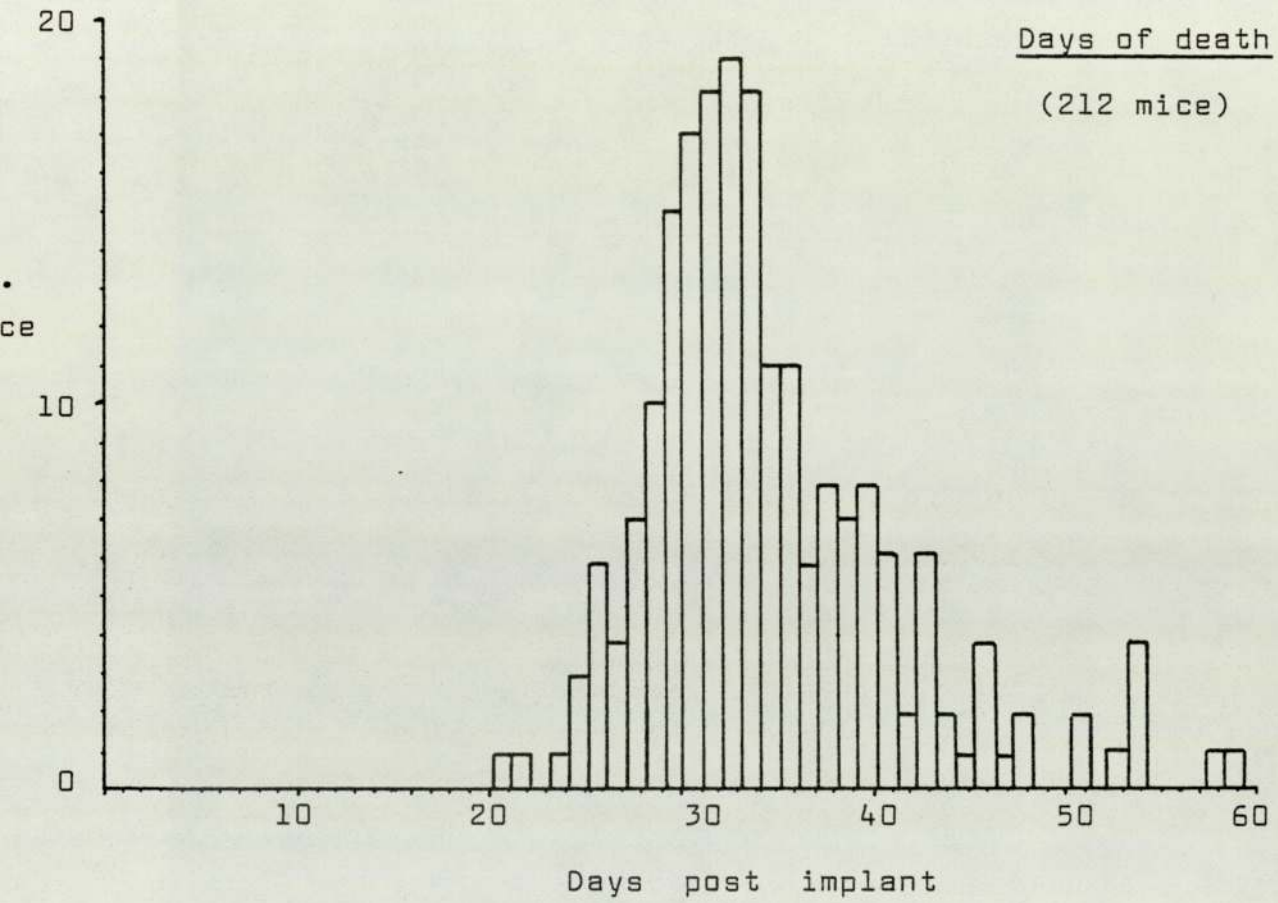


Fig. 6.1 Lifespan of BDF_1 ♀ mice implanted i.m. with 10^6 M5 sarcoma cells

Group	No.mice	Median day of death	Range of days of death
A	30	39.0	25 - 58
B	27	32.4	26 - 59
C	30	32.7	21 - 46
D	32	32.9	24 - 54
E	28	33.3	26 - 46
F	28	35.5	25 - 51
G	37	31.3	26 - 48
	212	31.3 - 39.0	21 - 59

Table 6.1 The days of death of mice inoculated with 10^6 M5 sarcoma cells.

The spread of the death days is comparatively tight for a slow growing tumour and the range of medians for several experiments narrow enough to suggest that the M5 sarcoma might be used as a survival time model. Death is through metastasis, however immediately prior to this, tumours are very large (approx. 7g) and have often broken through the skin of the leg leading to ulceration and infection. These problems militate against the use of this particular end point. Survival time is also a measure more of metastatic potential than of solid tumour growth.

Tumour weights are more accurate than tumour volume measurements, however, since the tumour must be excised for the former it is necessary to kill the mouse and terminate the experiment. The tumour volume ratios for treated to control mice were found to be almost identical to the tumour weight

ratios. Thus although it is difficult to measure absolute tumour volumes accurately, if one volume is divided by another an accurate ratio can be obtained. Tumour volume measurements are rapid and may be made as many times as required permitting the progress of growth to be monitored. In fig. 6.2 (appendix 6.1) the tumour volumes with respect to time are shown for groups of mice implanted with differing numbers of M5 cells. These coincide well with the growth curves of the M5 sarcoma implanted in C57/BL mice,[160] giving an indication that the BDF_1 mouse is an acceptable host for this tumour. The delay times to reach the arbitrary mean tumour volume of 3.5 cm^3 are recorded in table 6.2 as this parameter was used as a measure of tumour volume inhibition in some experiments.

Number of cells implanted	Days to reach 3.5 cm^3	Delay relative to 10^6 cells
10^6	20.5	-
10^5	32.5	12.0
10^4	40.8	20.3
10^3	47.8	27.3
10^2	53.6	33.1

Table 6.2

Several parameters of the M5 sarcoma are summarized in fig. 6.3 and table 6.3 and these are compared with those of other well established tumour model systems. Data for the L1210 leukaemia, B16 melanoma and Lewis Lung carcinoma systems were obtained from reference 163. The cell cycle time, S-phase time and proliferative fraction for the M5

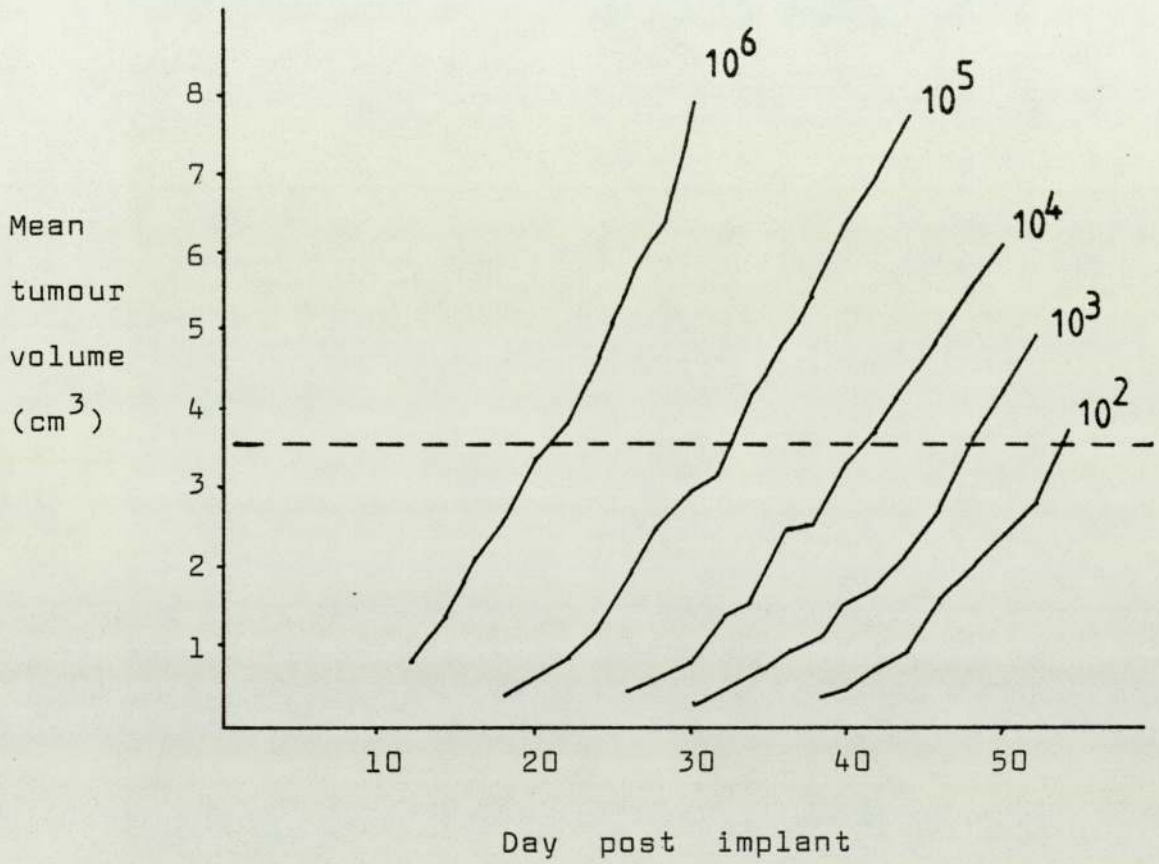


Fig. 6.2 Growth curves of the M5 sarcoma after implantation (i.m.) of differing numbers of tumour cells in BDF_1 ♀ mice.

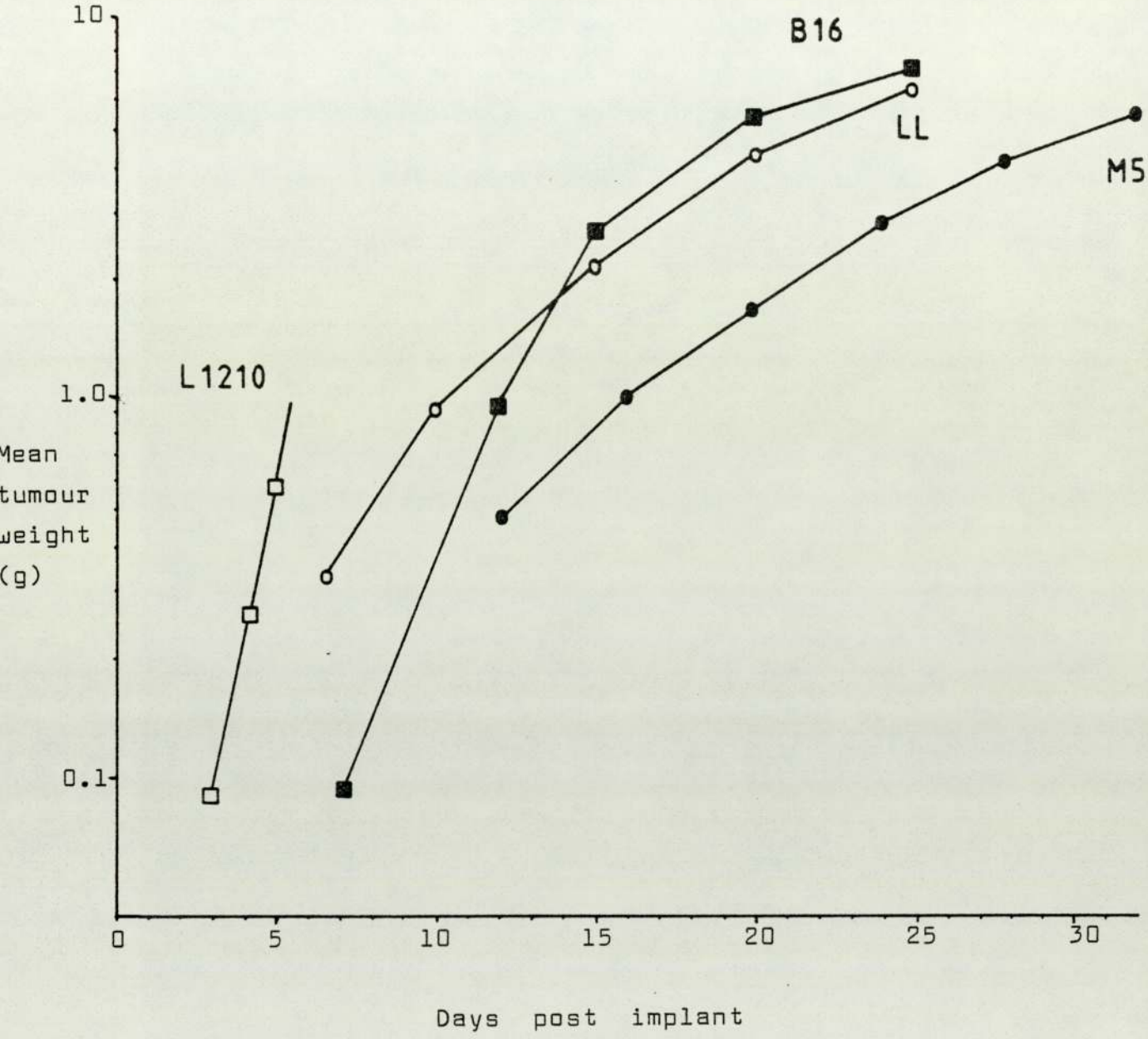


Fig. 6.3 Comparison of the growth rate of the M5 sarcoma with other murine tumour models.

sarcoma were reported by Simpson-Herren et al.[157] The growth curve and doubling time were determined for the 212 mice in table 6.1

Tumour	Site	Tumour size	Cell cycle time (h)	Time of s-phase (h)	Proliferative fraction (%)	Doubling Time (days)
L1210 leukaemia	i.p.	10^6 cells	12.8	9.0	86	0.5
B16 melanoma	s.c.	560mg	20.0	7.0	55	1.9
Lewis lung carcinoma	s.c	575mg	19.0	8.5	38	2.9
M5 sarcoma	i.m.	500mg	24-30	15-18	$\geqslant 40$	4.0

Table 6.3 Cell cycle and growth parameters of the experimental tumours

The data indicates that the M5 sarcoma is one of the slowest growing experimental murine tumours (the Lewis lung carcinoma is considered to be relatively slow). There is a need for slow growing experimental solid tumours with low proliferative fractions since these are the characteristics of the most intractable human neoplasms. Most novel agents are still tested on experimental leukaemias and lymphomas with short cell cycle times and high proliferative fractions, a situation which can be considered to have at least led to some progress in the treatment of human leukaemias and lymphomas but comparatively little with respect to the treatment of low proliferative tumours.

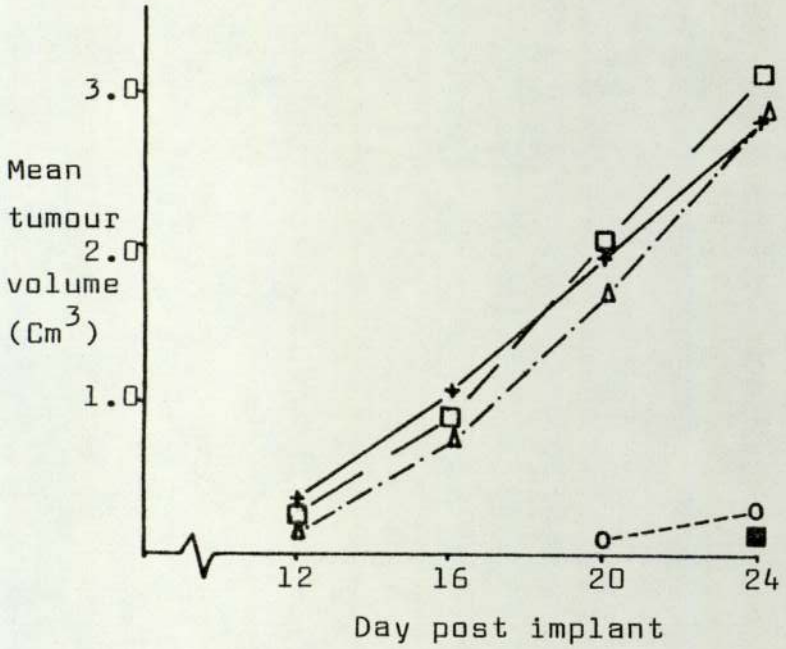
Mention has already been made of the metastatic spread of this tumour. It was observed at autopsy that the ovaries of M5-tumoured mice were often grossly enlarged through metastasis. Whereas normal ovaries weigh 3-5 mg, those in mice with advanced M5 sarcomas often weighed over 500 mg. This suggested that the weight of the ovaries might be employed as a simple model for metastasis. However investigations of a large number of mice with very advanced primary M5 tumours revealed ovaries weighing anywhere between 3 mg and 550 mg. Thus metastasis to this organ appears to be too inconsistent to be used in this way to quantitatively model for metastases.

6.3 Response of the M5 sarcoma to chemotherapy

Brief mention has been made by several authors of the response of the M5 sarcoma to chemotherapy, however few details have been published.[157,164,165] A variety of clinical drugs representative of different classes of agents were therefore tested against the M5 sarcoma to determine its spectrum of activity. In order to be able to compare different agents a therapeutic index (TI) was defined as the ratio of the LD_{50} dose to the dose required to produce 90% inhibition (ID_{90}) of the control tumour volume on day 24 after implant of the tumour.

The results are illustrated in fig. 6.4 and tabulated in table 6.4 (full details are given in appendix 6.2)

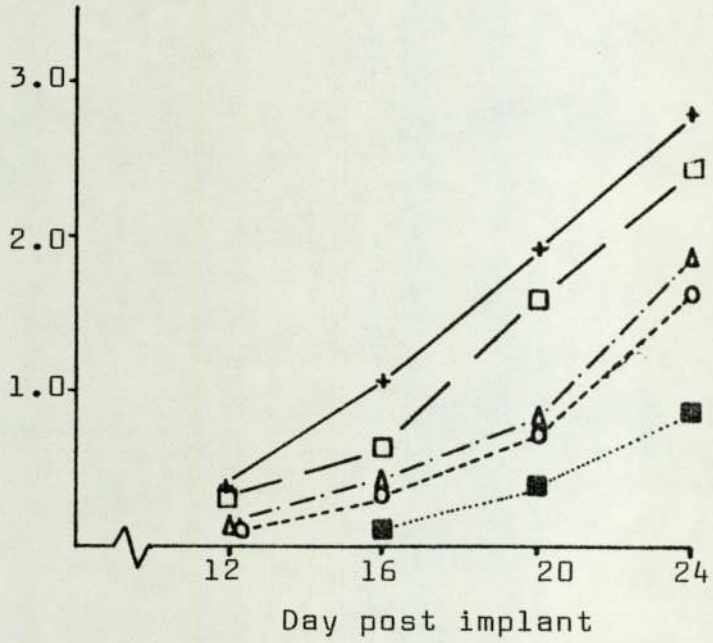
CYCLOPHOSPHAMIDE



Schedule : Day 1 only

- 200 mg/kg/day
- 100
- 50
- 25
- Control

CHLORAMBUCIL

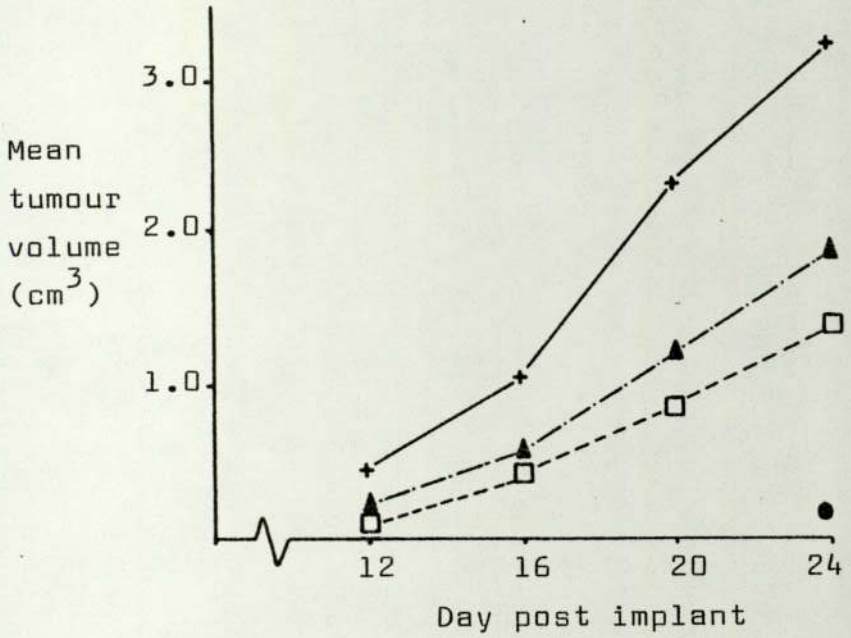


Schedule : Day 1 only

- 50 mg/kg/day
- 25
- 12.5
- 6.25
- Control

Fig. 6.4 Chemosensitivity of the M5 sarcoma

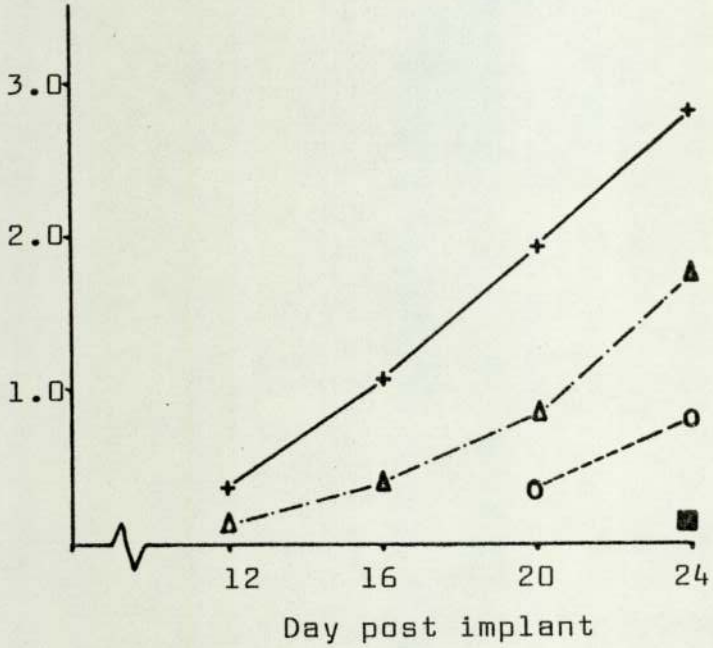
CIS-DIAMMINEDICHLOROPLATINUM (II)



Schedule: Days 1,5,9,13,17

4mg/kg/4days
2
1
Control

CCNU



Schedule: Day 1 only

20mg/kg/day
10
5
Control

Fig. 6.4 Chemosensitivity of the M5 sarcoma (cont)

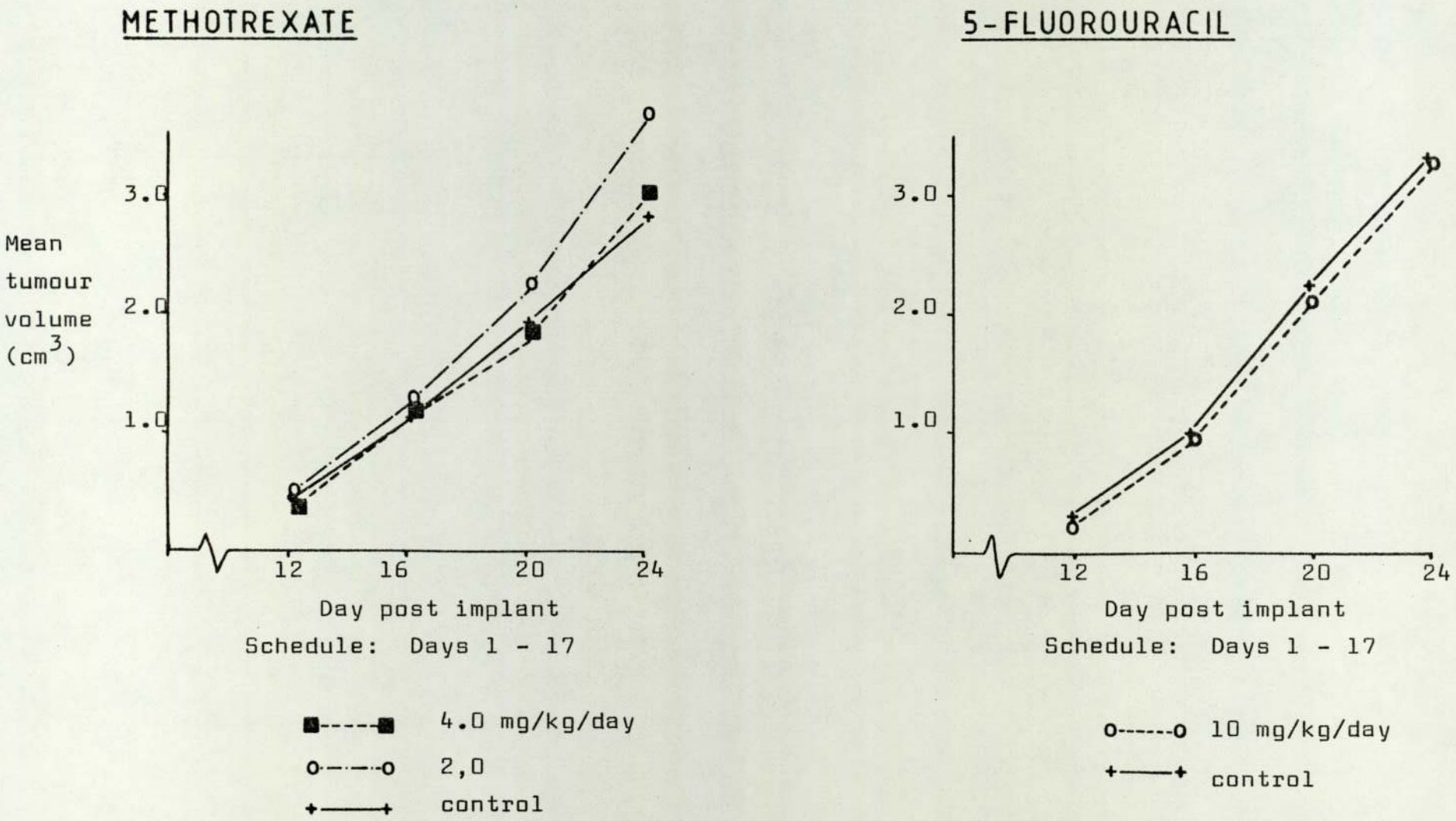


Fig. 6.4 Chemosensitivity of the M5 sarcoma

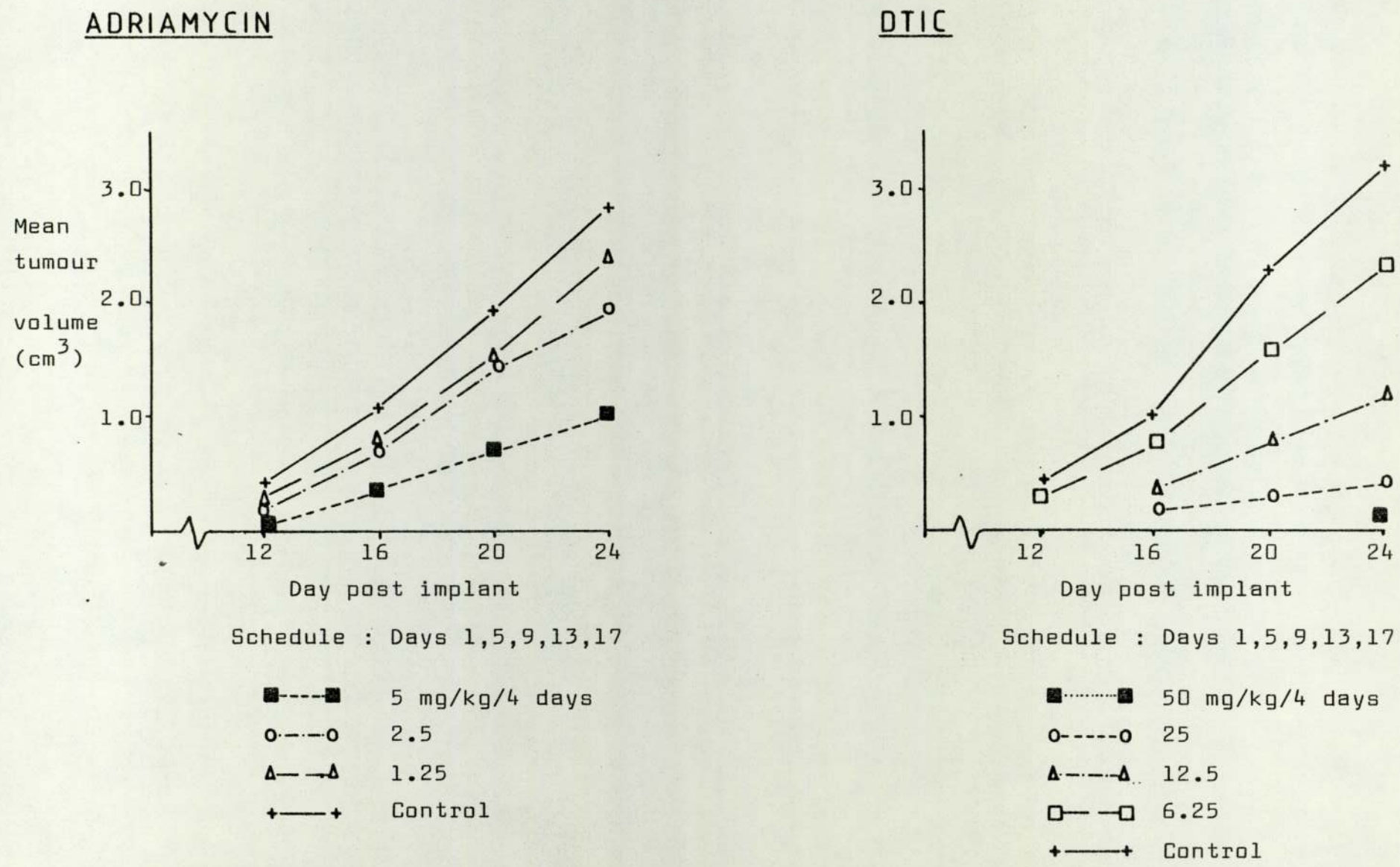
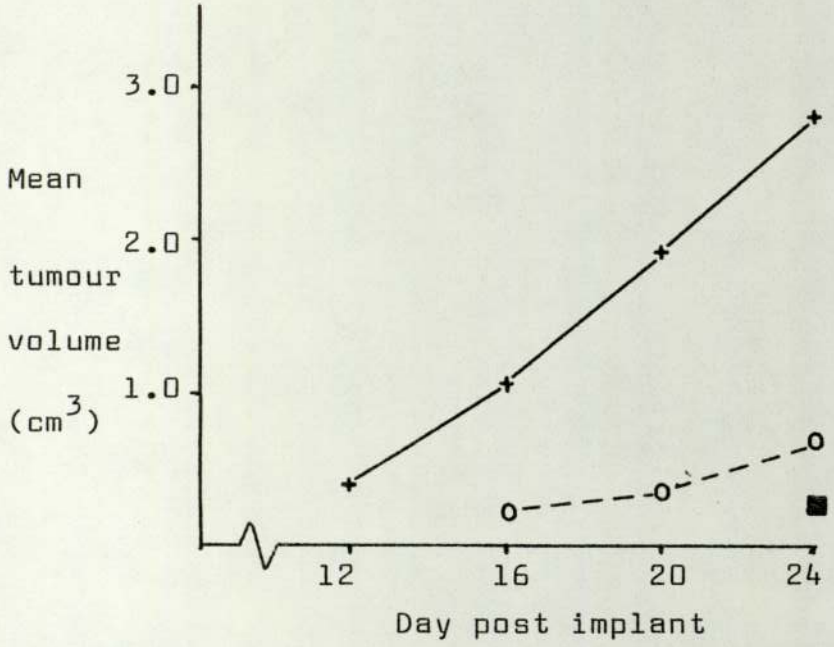


Fig. 6.4 Chemosensitivity of the M5 sarcoma (cont)

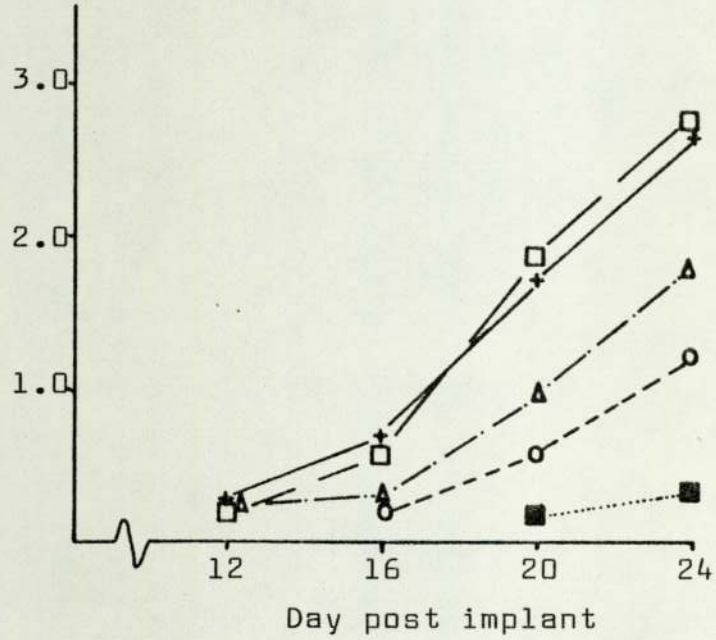
PROCARBAZINE



Schedule : Days 1,5,9,13,17

25 mg/kg/4days
12.5
Control

TREOSULPHAN



Schedule : Days 1 - 17

400 mg/kg/day
200
100
50
Control

Fig. 6.4 Chemosensitivity of the M5 sarcoma (cont)

* = mg $kg^{-1}day^{-1}$

Compound	Schedule (days)	LD_{10}^{*}	LD_{50}^{*}	ID_{90}^{*}	TI
Cyclophosphamide	1 only	370	520	100	5.2
Chlorambucil	1 only	26	45	~ 97	(~ 0.4)
CDDP	1,5,9,13,17	6.3	7.3	3.6	2.0
CCNU	1 only	43	57	17	3.4
Methotrexate	1 - 17	2.6	3.8	Inactive	Inactive
5-Fluorouracil	1 - 17	10.7	14.2	Inactive	Inactive
Adriamycin	1,5,9,13,17	3.7	5.6	(~ 10.2)	(~ 0.5)
DTIC	1,5,9,13,17	185	260	32	8.1
Procarbazine	1,5,9,13,17	240	470	23	20.4
Treosulphan	1 - 17	570	>800	420	⩾ 1.9
Hexamethylmelamine	1 - 17	99	110	69	1.6

Table 6.4 Chemosensitivity of the M5 sarcoma

The studies which have previously examined the chemosensitivity of the M5 sarcoma were conducted to determine whether the tumour might predict agents active against ovarian tumours.[157,164] Since the tumour was originally thought to be a carcinoma, the histology of 75-90% of human ovarian tumours,[166] it was proposed that it might model the pattern of chemosensitivity in the human situation. This, with the benefit of hindsight, is now known to have been a dubious speculation since the neoplasm is not a carcinoma but a reticulum cell sarcoma. As a predictive system the results in table 6.4 indicate that the tumour models well for the alkylating agents including treosulphan; overpredicts for CCNU, DTIC and procarbazine and is

incorrect for the antimetabolites. The M5 sarcoma would thus fare no better than any other experimental system. The responsiveness of the tumour is wide enough to detect agents such as HMM and treosulphan which are generally inactive towards other murine models.

The concept of an experimental tumour predicting for the clinical tumour of equivalent histology is at best optimistic. Since a compound might be regarded as active in the clinic when only 25% of patients respond then it is more likely to be inactive rather than active against the experimental model if a correlation were expected. Thus, for instance, a model for the highly intractable small cell carcinoma of the lung should predict none of the currently available drugs as active since no single agent will produce more than about a 41% overall response rate in lung cancer patients.[167]

Chapter 7 Antitumour studies of analogues of hexamethylmelamine (HMM)

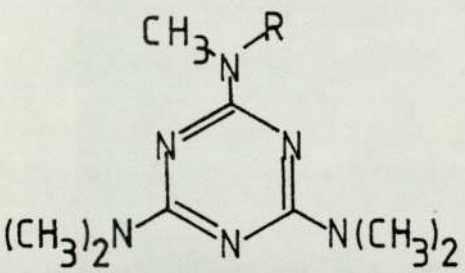
7.1 Introduction

The antitumour properties of HMM were first demonstrated upon the murine Sarcoma 180[168] and the Walker 256[134] rat carcinoma models. Activity has now also been shown against the Lieberman plasma cell[169] and the ADJ/PC6A (PC6) plasmacytoma[170] systems. Compared to most other clinically employed agents the spectrum of activity of HMM against the experimental models is relatively narrow. Indeed, if HMM was synthesized today and tested upon only the N.C.I. panel of tumours, the system with which most novel compounds are now evaluated, then its antitumour potential might have remained undiscovered. It has now demonstrated very marked activity against several human tumour xenografts implanted in nude mice including lung,[171,172,173] mammary,[171] colon,[171,172] ovarian[172] and renal[172] neoplasms. Previous studies of HMM analogue testing have used the Sarcoma 180[91] and Lewis lung[91] carcinoma systems (comparatively unresponsive models) and the PC6 plasmacytoma[98,145] which to date has been the most sensitive murine model for this purpose.

The activity of both HMM[157,169] and pentamethylmelamine (PMM)[169] against the M5 sarcoma has been reported and so this tumour was used for the study of further analogues of these compounds.

7.2 Schedule dependency of the methylmelamines

A previous investigation of the schedule dependency of PMM against the M5 sarcoma revealed that for this agent chronic long term schedules were superior to acute or intermittent regimes.[169] Since a number of methylmelamines and related structures were to be screened, a schedule was required with which they might be compared. Three possible chronic schedules (table 7.1, schedules A - C) were investigated for three melamines (12, 33, 37) and a fourth was investigated for HMM alone (table 7.1, schedule D).



(12) R = CH_3

(33) R = H

(37) R = CH_2OH

	Schedule
A	daily 1 - 17
B	days 1,3,5,7,9,11,13,15,17,19,21
C	days 1,3,5,7,9,11
D	days 1,5,9 (8 times/day)

Table 7.1 Chronic schedules compared for the melamines

In figs 7.1 approximately equitoxic doses ($\sim LD_{10}$ values) are contrasted for each schedule with each of the melamines (appendix 7.1). The comparison revealed that schedules A and B were equivalent at equitoxic dose levels whereas schedule C was undoubtedly inferior. Schedule C is merely a shortened version of schedule B, however higher doses are no more tolerable. Schedule D proved to be very toxic.

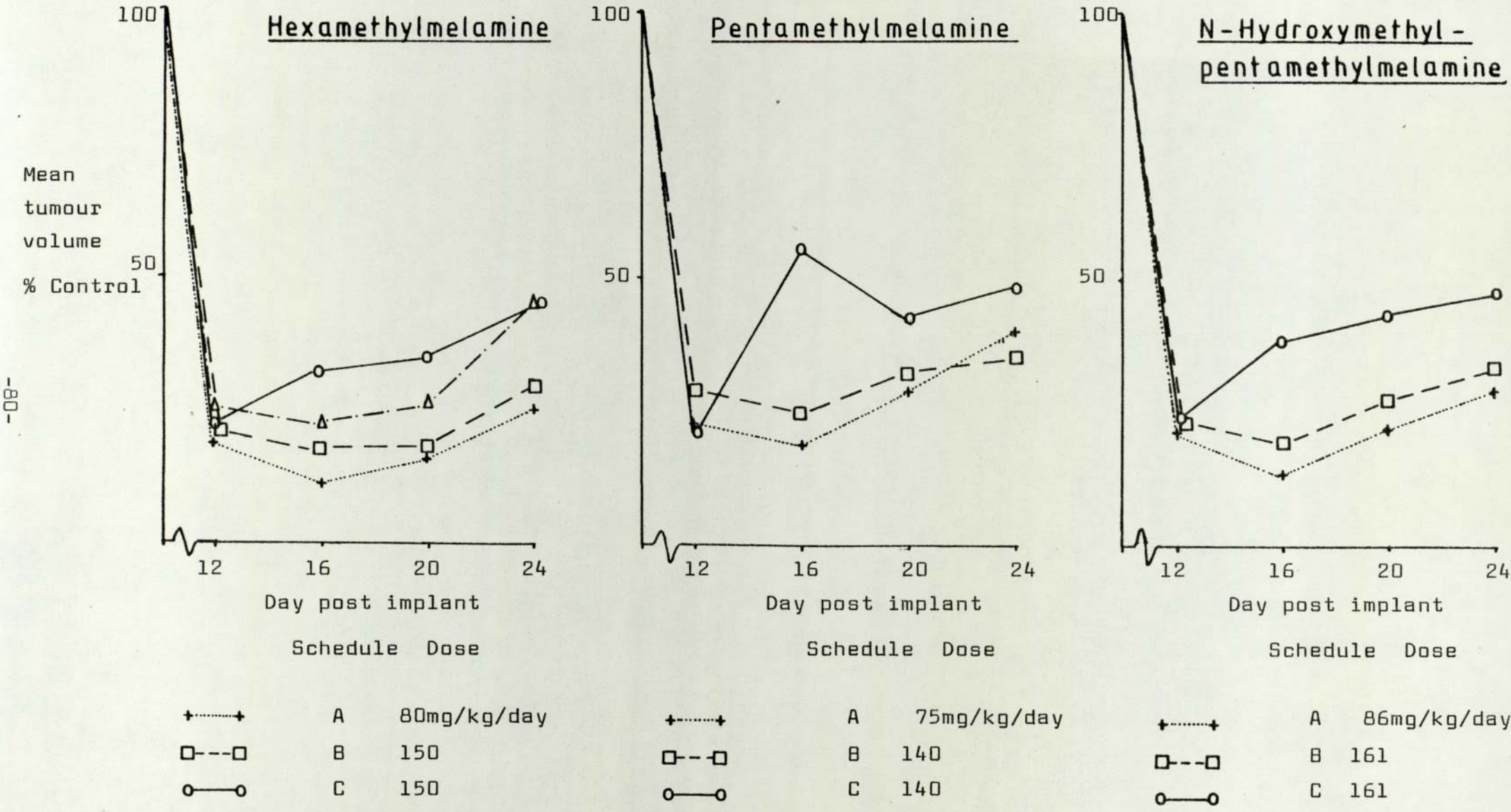
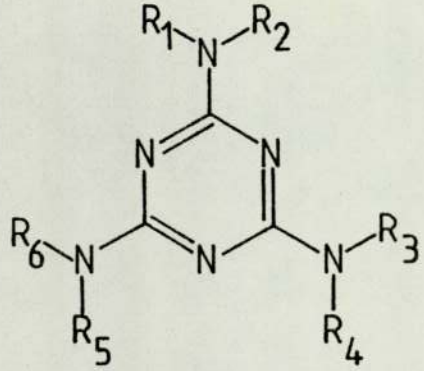


Fig. 7.1 Schedule dependency of some methylmelamines

Schedule A was therefore selected for general screening, however some of the initial tests were conducted using schedule B.

7.3 A comparison of the alkylmelamines against the M5 sarcoma

Two studies have already investigated the activities of the methylmelamines against experimental tumours, but differences appear to exist between the models. Lake et al.[91] in his study using the Lewis lung carcinoma and Sarcoma 180 systems concluded that the methylmelamines containing from six to two methyl groups all have similar therapeutic indices and thus the presence of an N-methyl group was sufficient for activity although monomethylmelamine was significantly less effective. Rutty et al. using the PC6 tumour showed that the methylmelamines containing from four to six methyl groups were active, however N^2,N^4,N^6-trimethylmelamine (triMM) and its N-demethylated homologues were completely inactive.[98] In addition HMM demonstrated activity superior to the other methylmelamines. The results for several alkylmelamines tested against the M5 sarcoma are shown in table 7.2 (appendix 7.2). They are in agreement with those obtained against the PC6 tumour although HMM was not found to be markedly superior to either PMM or N^2,N^2,N^4,N^4-tetramethylmelamine (TMM). TriMM was inactive even at its LD_{10} dose (~20% inhibition). M and HEM were inactive confirming the requirement of the N-methyl group for activity.



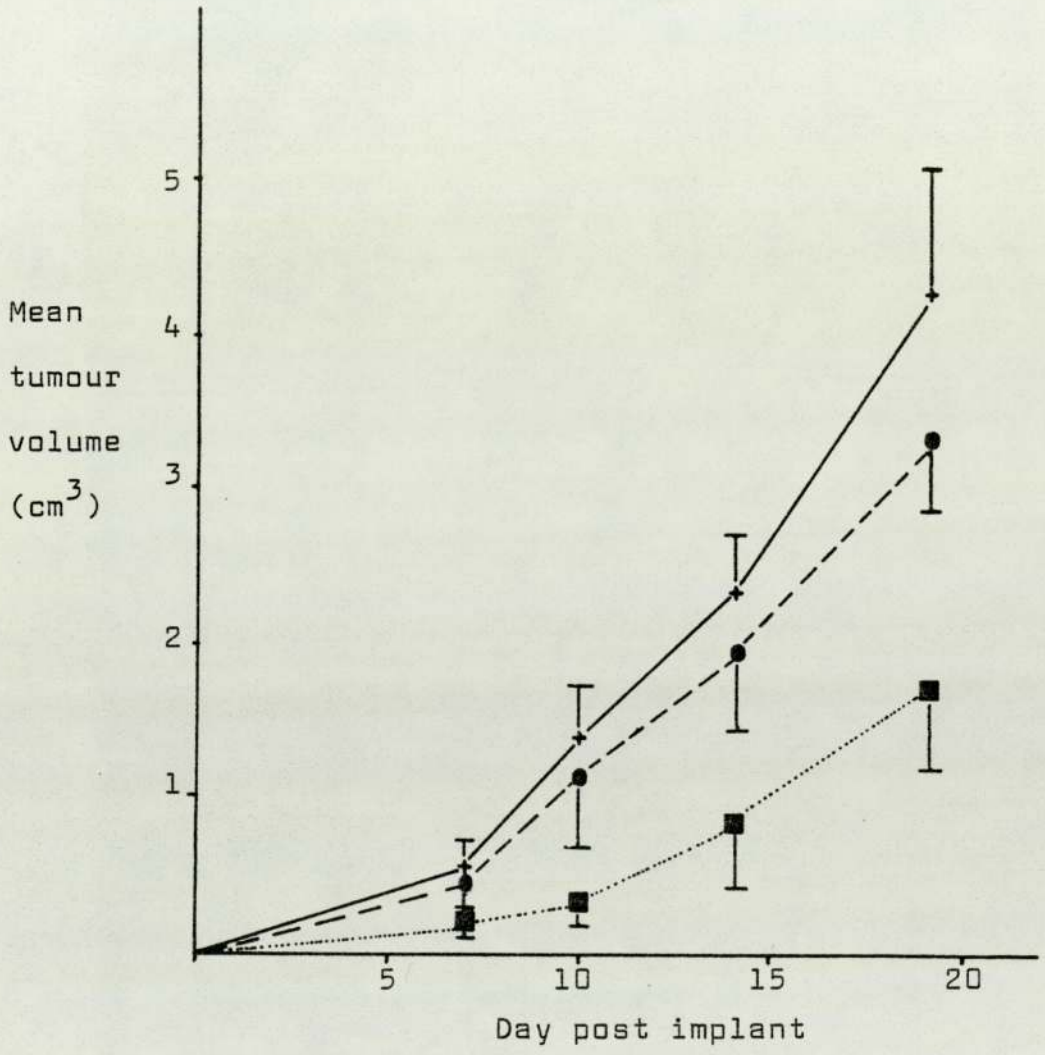
COMPOUND		R_1	R_2	R_3	R_4	R_5	R_6	LD_{10} *	LD_{50} *	ID_{90} *	TI
Hexamethylmelamine	HMM	CH_3	CH_3	CH_3	CH_3	CH_3	CH_3	99	110	69	1.6
Pentamethylmelamine	PMM	CH_3	H	CH_3	CH_3	CH_3	CH_3	144	167	132	1.3
Tetramethylmelamine	TMM	H	H	CH_3	CH_3	CH_3	CH_3	154	180	155	1.2
Trimethylmelamine	TriMM	CH_3	H	CH_3	H	CH_3	H	170	225	-	Inactive
Melamine	M	H	H	H	H	H	H	960	1180	-	Inactive
Hexaethylmelamine	HEM	C_2H_5	C_2H_5	C_2H_5	C_2H_5	C_2H_5	C_2H_5	820	-	-	Inactive

* = mg kg^{-1} day^{-1}

Table 7.2 Activity of the alkylmelamines against the M5 sarcoma.

Both were very much less toxic than the methylmelamines tested. HEM and HMM are reported to be equitoxic to PC6 cells *in vitro* whereas the desmethyl homologues of HMM become progressively less cytotoxic.[100] Brindley *et al.* have shown that plasma levels of HEM and its metabolites reach only a fraction of those levels achieved by HMM and its respective metabolites after an equimolar dose of HMM implying a possible pharmacokinetic basis for the requirement of the N-methyl moiety.[106] Thus the nature of the alkyl substituent may determine the ability of the molecule to reach the tumour site and the number of methyl groups determine the cytotoxicity of the compound at the site.

Lake *et al.*[91] found that in his Sarcoma 180 model TriMM was as effective as HMM at equitoxic doses. In an attempt to repeat this result, HMM at 80 mg/kg/day over 9 days was compared with TriMM at 150 mg/kg/day using the same schedule. These can be considered equitoxic doses on the basis of LD_{10} values (both for a 17 day schedule and for a 5 day schedule) and on the observed body weight change which was the same for each agent. Although HMM produced the expected degree of activity, TriMM was without significant activity (fig.7.2 and appendix 7.3). An interesting parallel to the antitumour activity of the methylmelamines is in their ability to act as insect chemosterilants. Substitution of the hydrogen atoms in melamine by short chain alkyl groups containing less than three carbon atoms in a chain yields highly active chemosterilants for the house-fly *Musca domestica L.*[146]. However it is only the methylated melamines which are able to sterilize the male of the species. Oliver



Drugs were administered daily between days 1 and 9

HMM 80 mg/kg/day

TriMM 150 mg/kg/day

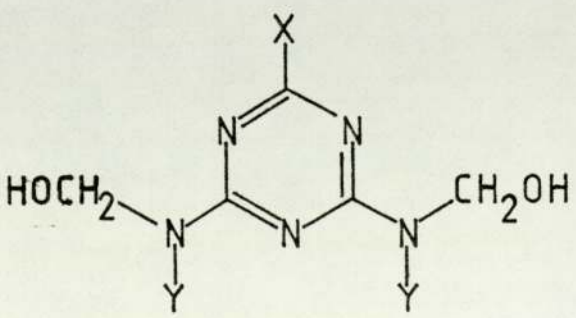
Control

Fig. 7.2 Activity of methylmelamines against the Sarcoma 180

et al. have suggested that the process of oxidative metabolism is probably responsible for the production of the active chemosterilizing species.[174]

It would appear, from the results in table 7.2, that there is not a great deal of difference in the therapeutic indices of HMM, PMM and TMM against the M5 sarcoma. If the N-methyl group is indeed directly responsible for antitumour activity, as seems the case, then only two dimethylamino groups appear to be necessary for activity which leaves room for potential modification in the third substituent position (59). The situation is not this clear cut though since Cumber et al.[145] have demonstrated two N^2,N^4,N^6-trimethyl containing compounds to be active against the PC6 plasmacytoma (58).
(R,R',R" = CH_2OH or C_2H_5).

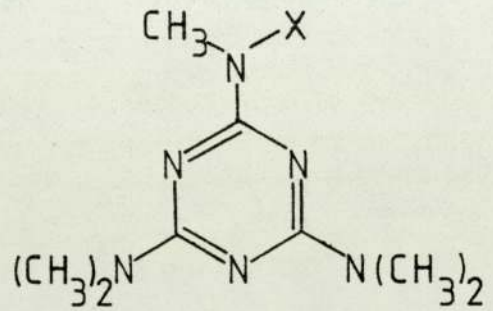
One might speculate that perhaps a dimethylol (81) is produced. The parallel between this structure and the general structure for an aziridinyl substituted s-triazine (53) active against the Walker 256 carcinoma[133] is obvious.



(81)

7.4 A study of the activity of N^2-substituted-N^2,N^4,N^4,N^6,N^6-pentamethylmelamine (N-substituted-PMM) compounds against the M5 sarcoma

The results for several N-substituted-PMM compounds tested against the M5 sarcoma are summarized in table 7.3 (Appendix 7.4).



Compound No.	X	LD_{10}	LD_{50}	ID_{90}	TI
12	$-CH_3$	99	110	69	1.6
33	-H	144	167	132	1.3
37	$-CH_2OH$	86	114	114	1.0
71	$-\overset{O}{\overset{\|}{C}}H$	95	187	198	0.9
66	-OH	350	494	Inactive	Inactive[a]
72	$-CH_2$-PMM[b]	~450	c		

a At 320 mg/kg/day, 27% inhibition (day 24)

b $N^2,N^{2'}$-Methylenebis(N^2,N^4,N^4,N^6,N^6-pentamethylmelamine)

c At 640 mg/kg/day, 59% inhibition (day 24, $^1/_5$ deaths)

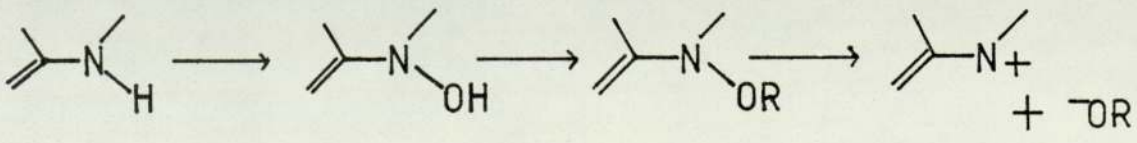
Table 7.3 The activity of N-substituted-PMM compounds against the M5 sarcoma.

The compounds tested represent some possible products after metabolism of just one methyl moiety in the HMM molecule. N-Hydroxymethylpentamethylmelamine (HMPMM; 37) is undoubtedly a major *in vitro* metabolite of HMM[101] and it is interesting that its toxicity and antitumour activity *in vivo* are not very different from those of HMM, although *in vitro* it is markedly more toxic.[100] This paradox is explicable by the fact that HMM is completely and very rapidly metabolized *in vivo* probably to this species which then undergoes further biotransformation.

N-Formyl metabolites have been detected in the metabolism of some N-methyl containing xenobiotics e.g. aminopyrine and N-methylbenzamide.[122]

N-Formylpentamethylmelamine (71) was active against both the M5 and PC6 tumours. However when sought for as an *in vitro* metabolite by g.c. analysis it was not found.[126]

N-Hydroxylation is a process which some aromatic amines and amides undergo to produce electrophilic nitrenium species (e.g. N-methyl-4-aminobenzene) (scheme 7.1)[175]



Scheme 7.1 N-Hydroxylation of amides.

It is possible that PMM might be N-hydroxylated and then esterified to produce a potentially electrophilic species. N-Hydroxypentamethylmelamine (66) however produced no significant inhibition against the M5 tumour although it is reported active against the PC6 plasmacytoma.[145]

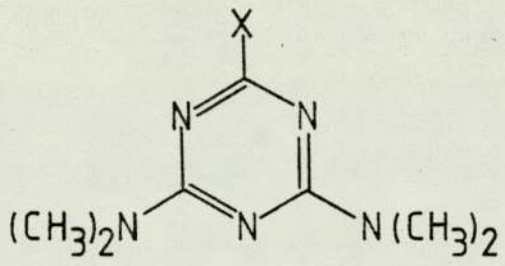
$N^2,N^{2'}$-Methylenebis(N^2,N^4,N^4,N^6,N^6-pentamethylmelamine; 72) is synthesized chemically by reaction of HMPMM (37) with PMM (33) (Scheme 5.2)

It is feasible that small amounts might be produced <u>in vivo</u> from this condensation. A small degree of activity was observed against the M5 sarcoma.

In conclusion it appears that N-substituted-PMM compounds are in general active however none of those tested was more active than PMM itself (other than the methyl substituted analogue, HMM).

7.5 A study of the activity of 2-substituted-4,6-bis (dimethylamino)-s-triazines against the M5 sarcoma

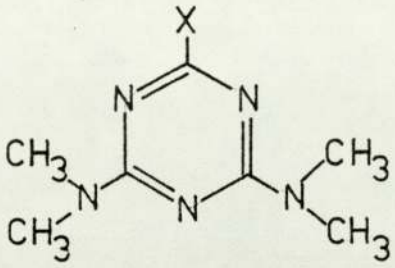
The results for several series of 2-substituted-4,6-bis(dimethylamino)-s-triazines are reported in tables 7.4 - 7.8. The optimal doses quoted are taken as those nearest to the LD_{10} values.

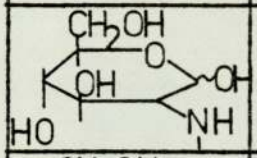
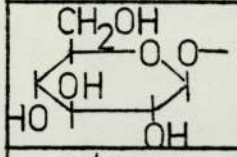
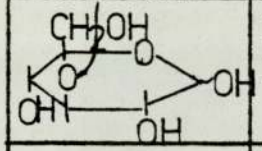


Compound No.	X	Abbreviation	Schedule (days)	LD_{10}	LD_{50}	Optimal dose mg/kg/day	No. Deaths / Total	% * Inhibition
62	$-NH_2$	TMM	1 - 17	154	180	160	$^1/_5$	92
64	-Cl	CBDT	1 - 17	135	270	160	$^1/_5$	4
67	$-NHNH_2$	HBDT	1 - 17	148	208	160	$^1/_5$	22
65	$-N_3$	ABDT	1 - 17	45	72	40	$^0/_5$	0

* day 24

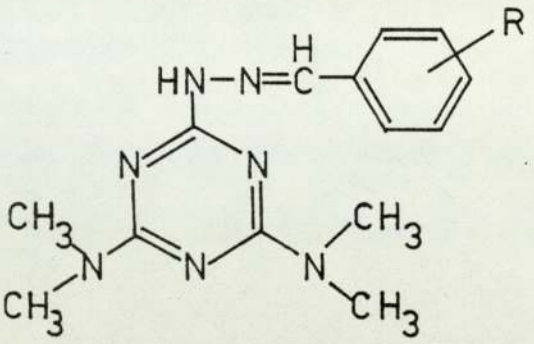
Table 7.4 Activity of 2-substituted-4,6-bis(dimethylamino)-s-triazines against the M5 sarcoma.



X	Schedule (days)	LD_{10}*	Highest* dose tested	No. deaths / Total	% Inhibition‡
	1,3,5,7,9,11, 13,15,17,19, 21	~200	200	1/10	0
	1,3,5,7,9,11, 13,15,17,19, 21	>300	300	0/10	0
	1,3,5,7,9,11, 13,15,17,19, 21	>300	300	0/10	0
$N(CH_3)_2$	1,3,5,7,9,11, 13,15,17,19, 21	~150	150	1/10	70

* $mgkg^{-1}2days^{-1}$ ‡ Day 24

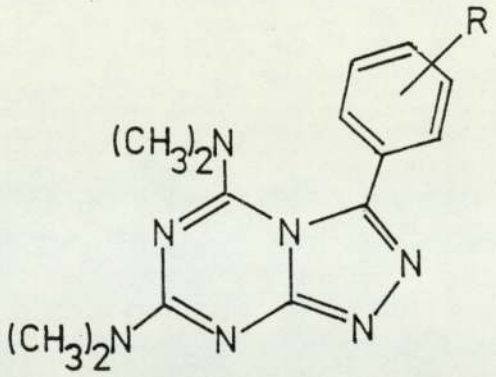
Table 7.5 Activity of the 2-sugar substituted-4,6-bis (dimethylamino)-s-triazines against the M5 sarcoma



R	Schedule (days)	LD_{10}*	Highest dose* tested	No. deaths / Total	%Inhi-‡ bition
-H	1,3,5,7,9,11,13, 15,17,19,21	>200	200	0/10	14
o-NO_2	1,3,5,7,9,11,13, 15,17,19,21	>200	200	0/10	12
p-NO_2	1,3,5,7,9,11,13, 15,17,19,21	>200	200	0/10	14
m-Cl	1,3,5,7,9,11,13, 15,17,19,21	>200	200	0/10	14
p-Cl	1,3,5,7,9,11,13, 15,17,19,21	>200	200	0/10	1

* $mgkg^{-1}2days^{-1}$ ‡ day 24

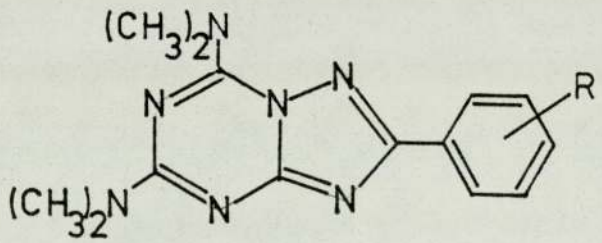
Table 7.6 Activity of the 2-substituted benzylidenehydrazino-4,6-bis(dimethylamino)-s-triazines against the M5 sarcoma.



R	Schedule (days)	LD_{10}*	Highest dose * tested	No. deaths / Total	% Inhi-‡ bition
-H	1,3,5,7,9,11,13, 15,17,19,21	>200	200	0/10	14
o-NO_2	1,3,5,7,9,11,13, 15,17,19,21	~200	200	1/10	6
o-Cl	1,3,5,7,9,11,13, 15,17,19,21	>300	300	0/10	29

* $mgkg^{-1}2days^{-1}$ ‡ day 24

Table 7.7 Activity of the s-triazolo [4,3-a]-1,3,5-triazines against the M5 sarcoma.



R	Schedule (days)	LD_{10}*	Highest dose * tested	No. deaths / Total	% Inhi-‡ bition
o-NO_2	1,3,5,7,9,11,13,15, 17,19,21	>200	200	0/10	4
o-Cl	1,3,5,7,9,11,13,15, 17,19,21	~300	300	1/10	26

* $mgkg^{-1}2days^{-1}$ ‡ day 24

Table 7.8 Activity of the s-triazolo [2,3-a]-1,3,5-triazines against the M5 sarcoma.

None of the 2-substituted-4,6-bis(dimethylamino)-*s*-triazine compounds tested were active except for the 2-amino compound (TMM) and those in table 7.3. Although TMM demonstrated significant activity at several dose levels, none of the other three compounds in table 7.4 showed the slightest activity even at lethal doses.

In addition, 2-hydrazino- (HBDT; 67) and 2-azido-4,6-bis(dimethylamino)-*s*-triazine (ABDT: 65) were shown to be completely inactive towards the PC6 tumour (appendix 7.5). ABDT is very much more toxic than others in the series. Ross has demonstrated that, whereas the other three compounds in table 7.4 all generate stable carbinolamines after microsomal activation *in vitro*, ABDT generates an unstable carbinolamine which rapidly decomposes to release formaldehyde.[176] If this rapid production of formaldehyde occurs *in vivo* it might well explain the enhanced toxicity of the compound.

The compounds in tables 7.5 - 7.8 were tested on the alternate day schedule, from days 1 - 21, which has been shown to be equivalent to the daily 1 - 17 schedule. The activity of HMM on this schedule is shown in table 7.5 for comparison.

2-Sugar substituted-4,6-bis(dimethylamino)-*s*-triazines although possessing the advantage of water solubility, were inactive at the doses tested (table 7.5 and appendix 7.6). The 2-substituted benzylidenehydrazino-4,6-bis(dimethylamino)-*s*-triazines (table 7.6 and appendix 7.7), intermediates in the

syntheses of the s-triazolo-1,3,5-triazines (tables 7.7, 7.8 and appendix 7.8) were also inactive at the dose levels tested. Although few of these agents reached lethal levels, there were no indications of activity at the highest doses tested.

The compounds in tables 7.4, 7.6 and 7.7 were also tested against the P388 leukaemia but were all found to be inactive (appendix 7.9).

The complete inactivity of HBDT relative to TMM demonstrates that tight structural requirements for activity exist for this class of antitumour agent.

Since previous studies have indicated that the abilities of these compounds to be demethylated may be related to their antitumour activity[98] the metabolism of some of these compounds was investigated.

Chapter 8 Metabolism and cytotoxicity studies of analogues of hexamethylmelamine (HMM)

8.1 Introduction

Studies by Cumber *et al.*[145] and Rutty *et al.*[98] have already revealed that some 2-substituted-4,6-bis(dimethylamino)-*s*-triazines are demethylated *in vitro*. Although Cumber *et al.*[145] considered no correlation existed between this parameter and antitumour activity *in vivo* against the PC6 tumour, Rutty *et al.*[98] claimed the correlation was present. The above studies employed rat liver microsomes for the metabolic activations. Ross[176] has recently performed demethylation studies with microsomes obtained from the livers of balb/c mice and the values of demethylation rates he obtained are recorded in table 8.1 and listed relative to HMM. Correlations with antitumour activity do appear to exist, however there are some very conspicuous exceptions. Whereas C8DT (64) undergoes demethylation as efficiently as either HMM or PMM, it is totally inactive *in vivo*[98]. Also TMM demethylates only poorly and yet is almost as effective an antitumour agent as HMM or PMM *in vivo*.[98]

To compare *in vitro* demethylation with *in vivo* antitumour activity is a very large step. Comparisons of *in vitro* demethylation with *in vitro* cytotoxicity and *in vivo* demethylation with *in vivo* antitumour activity seemed potentially more fruitful aims and it was considered that these might shed some light on the above discrepancies.

8.2 The *in vitro* cytotoxicity of analogues of HMM

The *in vitro* cytotoxicities of three methylmelamines (12,33,62)

which are active towards both the M5 sarcoma and PC6 plasmacytoma were compared with those of TriMM (70), CBDT (64) and HBDT (67) which are inactive against the same tumours. The PC6 plasmacytoma was used for the cytotoxicity studies as the demethylation studies had been performed with livers obtained from balb/c mice, the host for this tumour.

PC6 cells were incubated with the compounds in the presence or absence of liver microsomal activation. The results of these experiments are recorded in table 8.1 alongside the demethylation values. The incubation time in these experiments was 2 hours.

Compound	Conc (mM)	Antitumour Activity		% Inhibition of PC6 cells		In vitro demethylation [c]
		M5 sarcoma	PC6 tumour	- Activation	+ Activation	
HMM	5	+[a]	+[b]	0	97	100
PMM	5	+	+[b]	0	>99	98
TMM	5	+	+[b]	0	60	38
TriMM	5	-	-[b]	0	3	6
CBDT	5	-	-	63	96	113
HBDT	5	-	-	24	11	13
HMPMM	5	+	+[b]	>99	>99	

a + = Active; - = Inactive

b Data of Rutty et al.[98]

c Data of Ross[176] (Rate relative to HMM)

Table 8.1 Comparison of in vitro cytoxicity with in vitro demethylation for selected analogues of HMM

Several conclusions can be drawn from this data. Firstly it is clear that microsomal activation is required to produce significant toxicity over a 2h time course *in vitro*, the exception to this being CBDT which is apparently cytotoxic before activation. The chloro group is reasonably labile and may be susceptible to substitution by bionucleophiles. Secondly the cytotoxicity produced upon microsomal activation correlates well with *in vitro* demethylation. Thus those compounds which demethylate most efficiently are the most cytotoxic. The anomalies remain however in that 'activated' CBDT is as cytotoxic as HMM after activation yet completely inactive as an antitumour agent *in vivo* whereas 'activated' TMM is not as cytotoxic as HMM yet is as effective as HMM *in vivo*.

Either the carbinolamine or the formaldehyde generated may be responsible for this cytotoxicity. Ross has shown that all of these compounds form formaldehyde precursors (presumably the carbinolamine) which are stable for a period of at least $\frac{1}{2}$h.[176] However the duration of these incubations was 2h and it is likely that both the carbinolamines and formaldehyde were present and thus both might contribute to the cytotoxicity. It is also probable that more than one carbinolamine will be represented if demethylation produces more than one desmethyl analogue.

When HMPMM, the carbinolamine generated from HMM, was incubated under the same conditions it proved to be cytotoxic

without the need for activation (table 8.1) confirming the findings of Rutty et al.[100] This was also the case for M5 cells incubated with the drug (table 8.2). Also using M5 cells the cytotoxicity of HMM upon activation was found to be dependent on the concentration of liver microsomes present.

Compound	Conc.of drug (mM)	Conc.of microsomes (mg ml^{-1})	% Inhibition of M5 cells	
			- Activation	+ Activation
HMM	5	200	16	58
	5	400	39	95
HMPMM	2.5	200	$\geq$ 99.9%	
	5	200	$>$ 99.9%	

Table 8.2 Cytoxicity of HMM and HMPMM against the M5 sarcoma

On the basis of these results and those of Rutty et al.[98] it appears that HMM requires metabolism to exert appreciable cytotoxicity. Rutty et al.[100] have shown that HMM is cytotoxic towards PC6 cells only after very prolonged exposure to the drug (e.g. 72h). D'Incalci has recently observed a small degree of covalent binding when labelled HMM is incubated with PC6 tumour microsomes suggesting that these microsomes may well have the ability to metabolize HMM.[105] It is feasible that this small degree of microsomal activation may be responsible for the cytotoxicity of HMM over a prolonged period. Rutty et al.[98,100] have demonstrated that the desmethyl analogues of HMM when considered on an equimolar basis are less active in vivo and less cytotoxic in vitro than HMM itself. Thus production of the desmethylmelamines

is likely to be a deactivation process and it is improbable that they represent the highly cytotoxic species generated from HMM.

Therefore the cytotoxic species generated from the methylmelamines, as the demethylation values suggest, are almost certainly the formaldehyde precursors (i.e. compounds which decompose to release formaldehyde under certain conditions) and/or free formaldehyde itself. It is reasonable to conjecture that the same species are the ones responsible *in vivo* for antitumour activity.

8.3 The *in vivo* metabolism of analogues of HMM

Since the cytoxicity of the methylmelamines correlated with the production of formaldehyde and formaldehyde precursors *in vitro* then it was of interest to see whether the antitumour activity might correlate with the production of these species *in vivo*. The three active methylmelamines and the three inactive analogues employed in the cytotoxicity studies were again used. Rutty *et al.*[124] have previously investigated the levels of formaldehyde-containing species derived *in vivo* from HMM and TriMM but were unable to differentiate between free formaldehyde and its precursors or to show a marked difference between the two compounds in the total levels generated. Formaldehyde precursor species have also been reported in the plasma of mice, rats and man after administration of PMM.[97]

An assay was required with the ability to differentiate between formaldehyde and its precursors. Two simple colorimetric assays are widely used for the determination of

formaldehyde. The Nash assay[177] is specific for formaldehyde, however assay conditions require heating at 60°C for 20 - 30 min. whereupon moderately stable precursors such as the carbinolamines of the methylmelamines are known to decompose, releasing their formaldehyde. Thus this simple assay would not differentiate between the two. Gescher et al.[120] have devised a method whereby free formaldehyde may be removed from the system by enzymic oxidation before the assay is conducted, thereby leaving only the precursors of formaldehyde to be detected. An alternative to the Nash assay is the 3-methyl-2-benzothiazolone hydrazone (MBTH) test.[178] This is an assay designed to detect water soluble aliphatic aldehydes. Ross has recently employed this assay to differentiate between formaldehyde and its precursors in the following manner.[122] At 0°C the assay is capable of detecting formaldehyde almost as efficiently as it detects it at 60°C. Since the formaldehyde precursors derived from all six of the HMM analogues under investigation are known to be stable at 37°C for periods of up to $\frac{1}{2}$h, they will certainly be stable at 0°C for the same time course.[176] Therefore if the assay reaction when conducted at 0°C determines no formaldehyde, then free formaldehyde is clearly not present. If then the assay when conducted at 60°C determines formaldehyde it may be presumed to originate from a precursor These principles are illustrated in fig. 8.1. The absorbance at 670 nm is used to monitor the product of the MBTH reaction with formaldehyde and this is shown to be proportional to standard amounts of formaldehyde or HMPMM added.

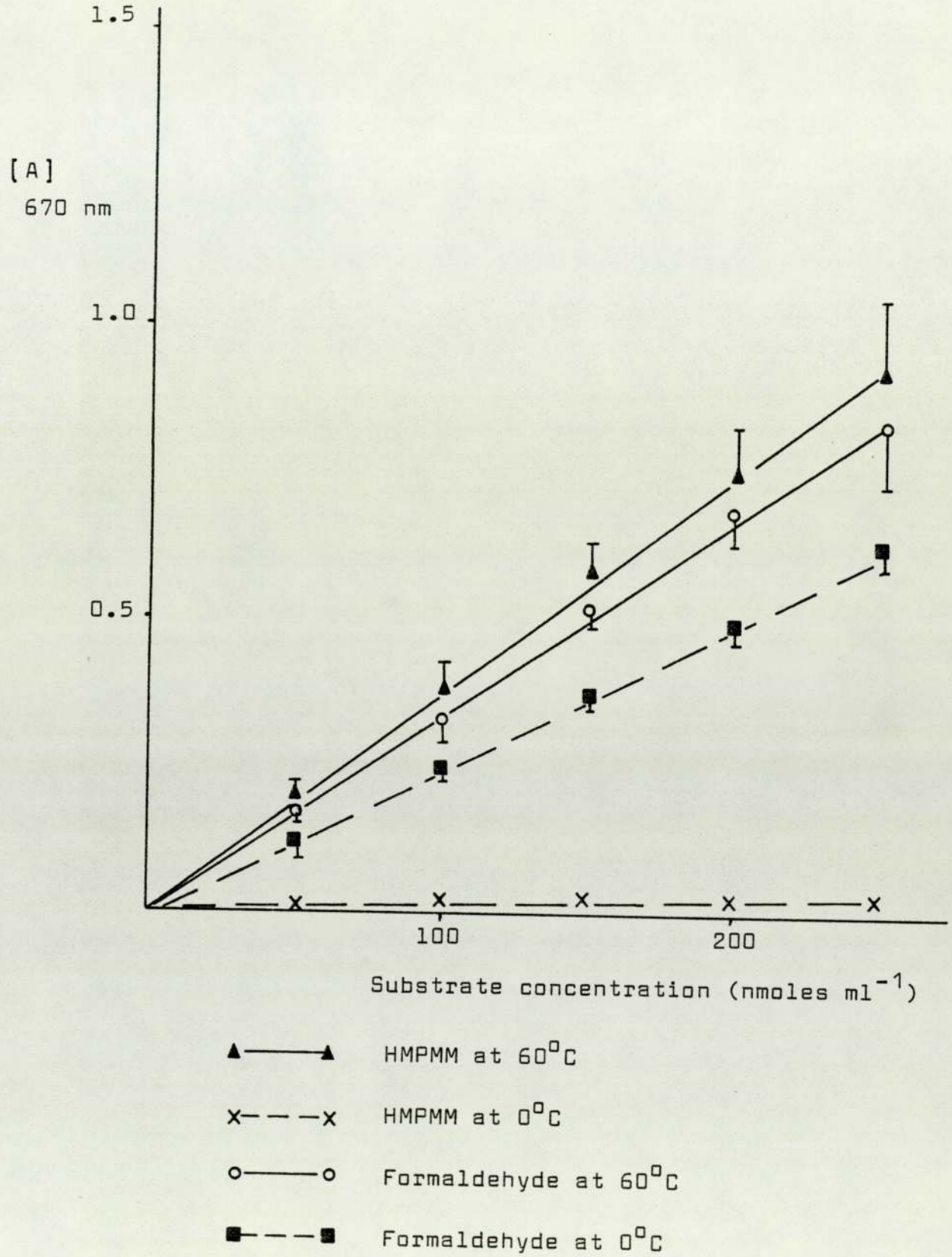


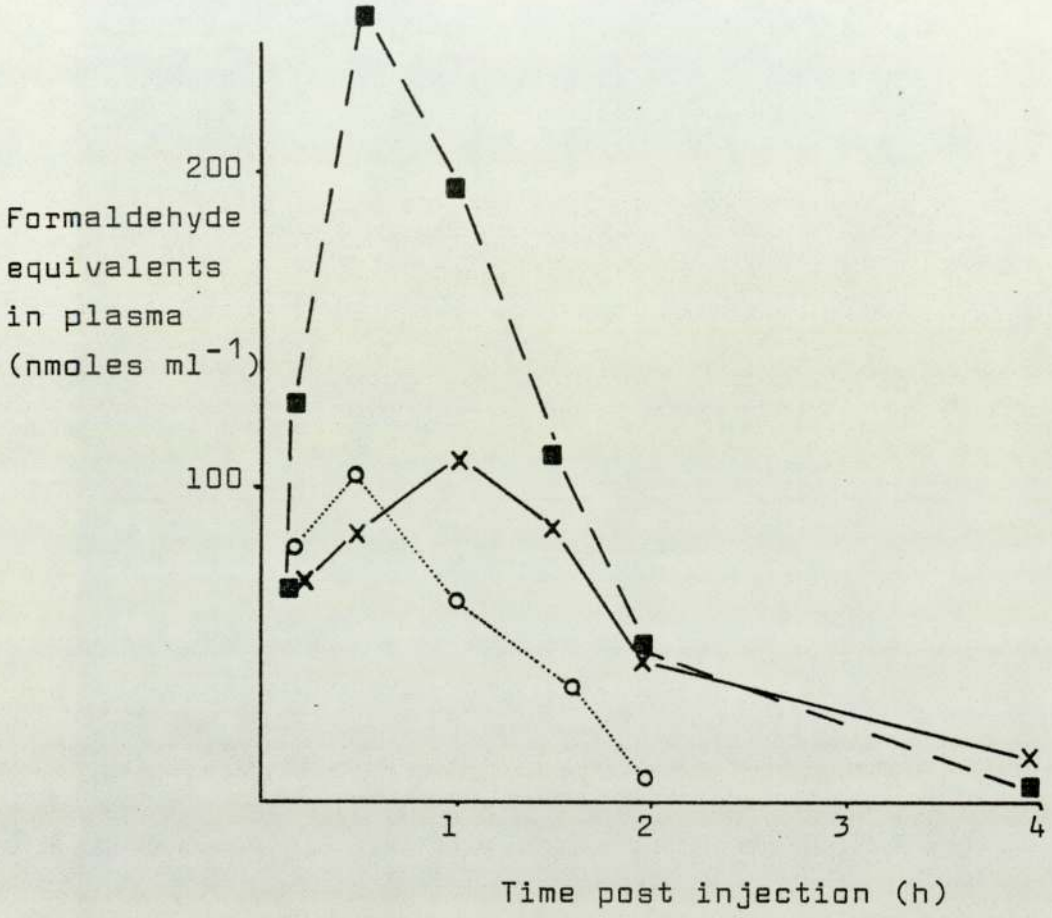
Fig. 8.1 Relationship between the absorbance at 670 nm of the product of the MBTH reaction and HMPMM and formaldehyde concentration

To investigate the levels of formaldehyde precursors and free formaldehyde in the plasma, balb/c mice were injected with 0.48 mmoles kg^{-1} of the drugs. This value corresponds to 100 mg kg^{-1} for HMM. At various time points blood was obtained from the mice by cardiac puncture and the levels of formaldehyde in the plasma were determined at $0^{o}C$ and $60^{o}C$ by the MBTH assay. The plasma levels obtained for the six compounds assayed are recorded in table 8.3. and illustrated in figs. 8.2 and 8.3. Only those levels of formaldehyde detected at $60^{o}C$ are illustrated since no significant levels were found when the assay was conducted at $0^{o}C$. This would imply that there are no significant levels of free formaldehyde generated by these compounds present within the plasma. Thus the levels are $60^{o}C$ are taken to correspond only to formaldehyde precursors. From fig.8.2 and table 8.3. it can be seen that the peak levels and total area under the curve values of the formaldehyde-containing metabolites of the three active methylmelamines are higher than those of the inactive analogues (appendix 8.1). The assumption is made that the precursor releases one mole of formaldehyde only.

Compound	Dose $mgkg^{-1}$	Antitumour Activity		Levels of formaldehyde precursors	
		M5 sarcoma	PC6 tumour	Peak level (nmoles ml^{-1})	AUC b (nmoles ml^{-1} x min
HMM	100	+[a]	+	111 (± 3)	14,580 (± 3,330)
PMM	93	+	+	243 (±27)	21,300 (± 6,300)
TMM	86	+	+	101 (±15)	7,530 (± 3,121)
TriMM	80	-	-	51 (±18)	3,060 (± 93)
CBDT	96	-	-	16 (±26)	96 (± 276)
HBDT	94	-	-	33 (± 9)	3,540 (± 810)

a + = Active; - = Inactive
b AUC = Area under the curve (± s.d.)

Table 8.3 Plasma levels of formaldehyde precursors generated by the analogues of HMM

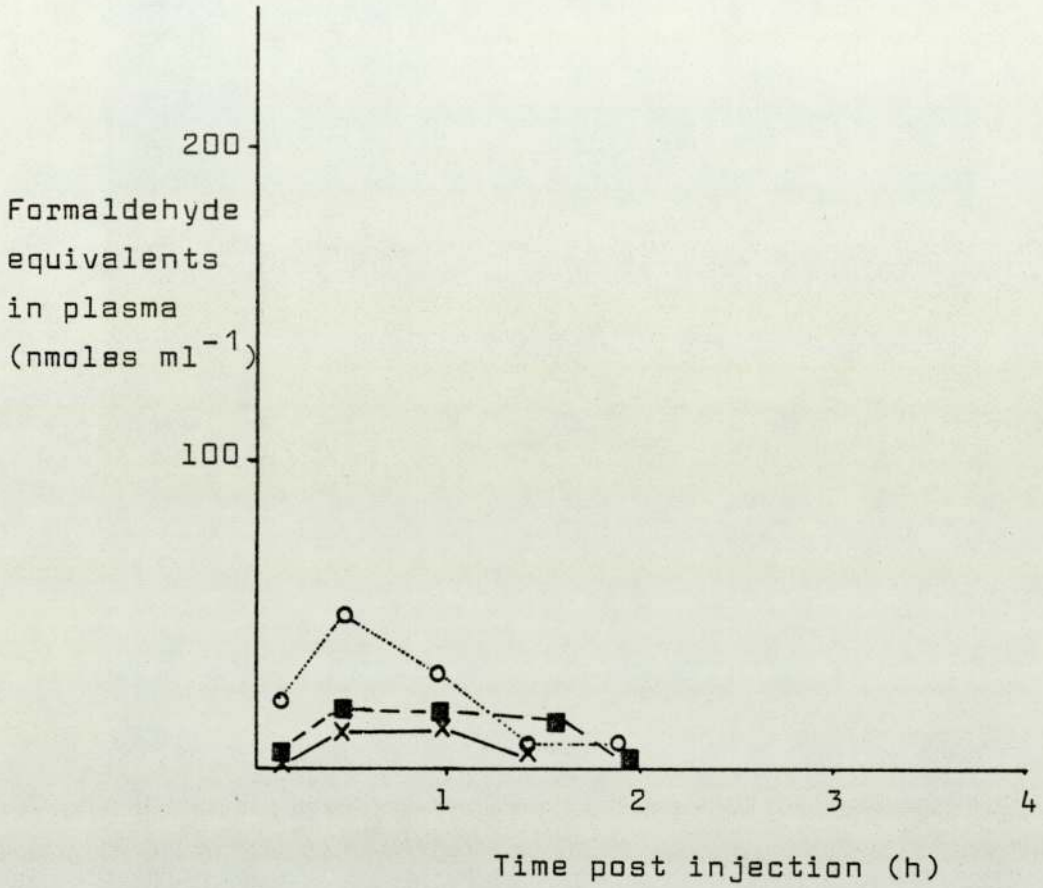


TMM 86 mgkg^{-1}

HMM 100 mgkg^{-1}

PMM 93 mgkg^{-1}

Fig. 8.2 Plasma levels of formaldehyde precursors generated by the analogues of HMM



TriMM 80 mgkg^{-1}

CBDT 96 mgkg^{-1}

HBDT 94 mgkg^{-1}

Fig. 8.3 Plasma levels of formaldehyde precursors generated by the analogues of HMM

It is likely that the formaldehyde-containing species being detected for each compound represent a range of metabolites. For instance, HMM within a 1h period is known to be demethylated to at least N^2,N^4-dimethylmelamine (scheme 1.4)[96], thus via a minimum of four different carbinolamines (or perhaps conjugates). Nothing is known of their relative cytotoxicities or stabilities or whether one might be specific for antitumour activity. The assumption is also made that these precursors all act similarly under the conditions of the MBTH assay. In support of this Ross has previously demonstrated that all of these compounds generate formaldehyde precursors which are stable at 37°C in solution yet decompose at 60°C to release their formaldehyde.[176]

To confirm that formaldehyde would be detected at 0°C if present, a series of control experiments were performed. Formaldehyde and HMPMM were separately added to either plasma, Earl's buffer or Earl's buffer with bovine serum albumin (B.S.A.) added (7 g/100 ml). The last solution was used to mimic the protein levels in plasma. The determination of formaldehyde from these two compounds in these solutions is indicated in table 8.4 and values are shown relative to the values obtained from Earl's buffer.

Compound	Conc. (μM)	Amount formaldehyde determined (%) *					
		Earl's buffer		Earl's buffer + B.S.A.		Plasma	
		0°C	60°C	0°C	60°C	0°C	60°C
Formaldehyde	300	100	100	89	89	79	78
HMPMM	300	N	100	N	47	N	63

* Values relative to those determined in Earl's buffer
N Negligible amount

Table 8.4 Recovery of formaldehyde and HMPMM from various solutions as indicated by the MBTH assay

Recovery of formaldehyde from either plasma or Earl's Buffer + B.S.A. proved to be less efficient than from Earl's buffer alone. These differences though were relatively small compared to those for the recovery of HMPMM from these solutions. The values of HMPMM (as measured by the amount of formaldehyde in the molecule) assayed in Earl's buffer + B.S.A. were markedly reduced suggesting possible protein binding which may also be the case in plasma. These controls indicate that formaldehyde is detectable at 0°C and also that the formaldehyde precursor levels obtained in vivo may well represent an underestimate of the actual amounts present.

With these qualifications in mind, several conclusions may tentatively be drawn from these experiments. Firstly, it is clear that there are no detectable levels of formaldehyde present within the plasma after administration of these compounds. Therefore it seems likely that formaldehyde may

only play a part if it is released from the formaldehyde precursors *in situ* at the tumour target. Secondly the levels of formaldehyde precursors generated from compounds active towards the PC6 and M5 tumours *in vivo* are markedly greater than those produced from the inactive compounds. Since the *in vitro* demethylation studies indicate these may well be the species responsible for *in vitro* cytotoxicity then it seems likely that they may also be responsible for the antitumour activity. This would explain why CBDT is inactive *in vivo* since only low levels of these metabolites appear in the plasma. However only a qualitative correlation can be claimed since PMM produces anomalously high levels of these species compared to either HMM and TMM yet possesses similar antitumour activity. Although PMM proceeds through a metabolic pathway analogous to HMM *in vivo* differences are known to exist. For instance, Colombo *et al.* have recently found an as yet unidentified conjugation product of PMM which has not yet been observed for HMM. It is feasible that these minor differences in metabolism may account in some way for the anomalously high values of formaldehyde precursors obtained from PMM.

On the basis of these results it is suggested that for a methylmelamine to be an antitumour agent it should have the ability to produce marked levels of formaldehyde precursors *in vivo* which are moreover bioavailable to the tumour. *In vitro* demethylation appears to be a useful indicator of the cytotoxicity of these metabolites but this is not the only parameter which will determine activity *in vivo*.

Thus the formaldehyde precursors deserve further study to elucidate their exact nature as they are eminently favourable candidates for the active species produced from the methylmelamines. Whether they are carbinolamines, conjugates of carbinolamines or some other species remains a matter of controversy. Furthermore the relatively greater plasma levels of these compounds derived from PMM in comparison to TMM without a consequent increase in antitumour activity might indicate that some are less important than others.

Although not found in the plasma the role of formaldehyde cannot be discounted since these molecules are obviously capable of releasing formaldehyde under appropriate conditions. The role of formaldehyde is investigated further in the following section.

8.4 The antitumour activity of formaldehyde

Formaldehyde has been shown to be cytotoxic to tumour cells *in vitro*.[98,100] It is implicated in the mechanism of action of HMM and so experiments were conducted in order to investigate its antitumour activity *in vivo*. Its activity towards several murine tumours is reported in table 8.5 and in appendix 8.2. Significant activity was demonstrated against the ascitic tumours including the P388 leukaemia and Sarcoma 180 (ascitic form) but not against the L1210 leukaemia or B16 melanoma. Indeed cures (no evidence of tumour on day 60) were obtained against the Sarcoma 180.

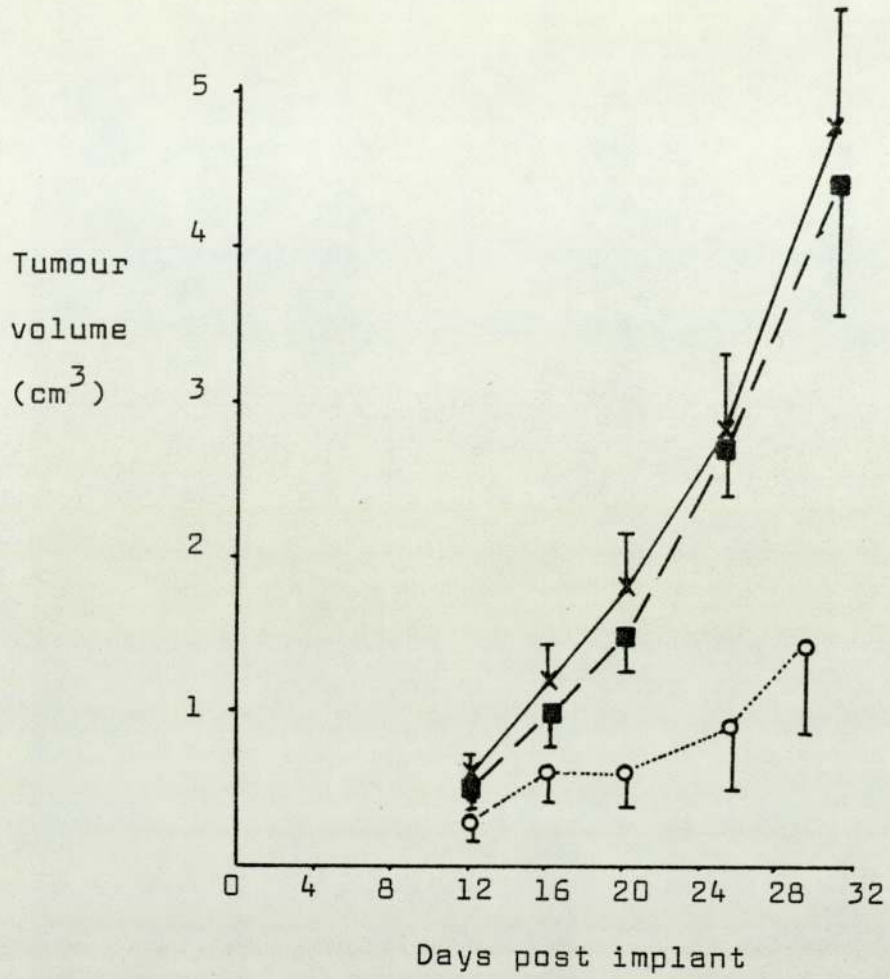
Tumour	Schedule Days of injection	Optimal dose $mg\ kg^{-1}\ day^{-1}$	Toxic Deaths / Total	% Increase in Survival time	No. Cures / Total
P388 leukaemia	Once daily 1 - 9	40	$^{0}/_{10}$	35	$^{0}/_{10}$
	Twice daily 1 - 9	25	$^{1}/_{10}$	32	$^{0}/_{10}$
	Three times daily 1 - 9	20	$^{0}/_{10}$	35	$^{0}/_{10}$
L1210 leukaemia	Once daily 1 - 8	40	$^{0}/_{10}$	18	$^{0}/_{10}$
B16 melanoma	Once daily 1 - 9	40	$^{0}/_{10}$	0	$^{0}/_{10}$
Sarcoma 180 (ascitic form)	Twice daily 1 - 5	30 30	3/16 0/8	48 41	3/16 3/8
	Once daily 1 - 5	35	$^{0}/_{10}$	0	$^{0}/_{10}$
	Once daily 1 - 9	30	$^{0}/_{10}$	78	$^{0}/_{10}$

Table 8.5 Activity of formaldehyde against several ascitic (i.p.) tumours

Significantly when tested against the Sarcoma 180 (i.m. solid form) and the M5 sarcoma (i.m. solid form) no tumour inhibition was found. This would suggest that formaldehyde is only acting when it is placed in immediate contact with tumour cells such as in an i.p. drug/i.p. tumour situation. Thus cures are possible when formaldehyde is injected directly

upon Sarcoma 180 cells within the peritoneal cavity, however the drug would appear to be detoxified (by formaldehyde and aldehyde dehydrogenases) before it reached tumour cells implanted intramuscularly within the leg. Although cures were obtained by giving the drug twice a day for five days against the ascitic Sarcoma 180, if given once daily for the same time course then no significant increase in survival time was obtained. Again this implies that levels of the drug must be very high to effect significant tumour inhibition.

To investigate further the ability of formaldehyde to 'travel' within a host, the following experiment was performed. M5 sarcoma cells were implanted intramuscularly into the left legs of BDF_1 mice. Formaldehyde was then injected intramuscularly into either the left leg (thus intratumourally) or the right leg (fig. 8.4 and appendix 8.3). Whereas tumour inhibition resulted when the drug was placed on top of the tumour cells, no effect was seen when the drug was injected at a distant site, i.e. the other leg. When the same experiment was performed with HMM (fig. 8.5 and appendix 8.4), identical inhibitions independent of the site of injection were elicited suggesting that HMM is not acting directly. This was further corroborated by an experiment employing the PC6 tumour wherein the tumour was implanted intramuscularly in the leg and HMM was injected by a variety of different routes (fig. 8.6 and appendix 8.5). The tumour inhibitions after a single injection of HMM (90 mg kg^{-1}) were all very



o·····o Formaldehyde 40 mgkg^{-1} day^{-1} (i.m.left leg)

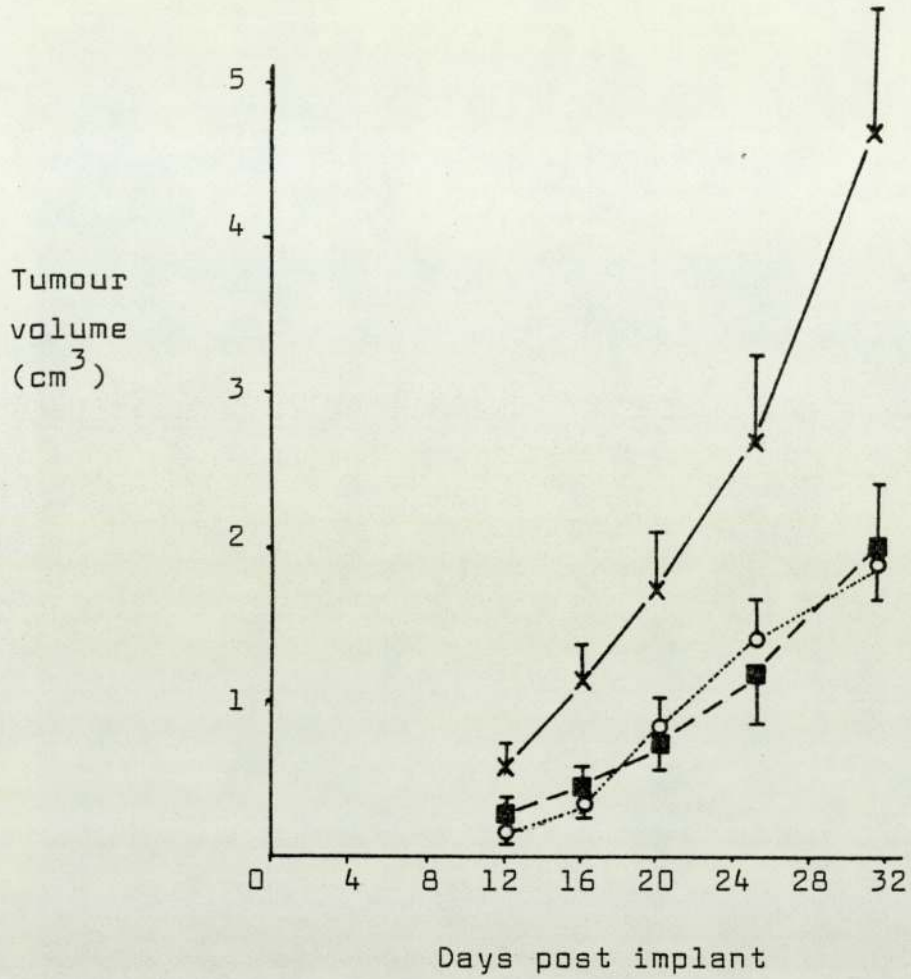
■----■ Formaldehyde 40 mgkg^{-1}day^{-1} (i.m.right leg)

x——x Control

Tumour implanted (i.m.) in left leg.

Treatment schedule days 1 - 5 : 13 - 17

Fig. 8.4 Formaldehyde against the M5 sarcoma



○·····○ HMM 80 $mgkg^{-1}day^{-1}$ (i.m.left leg)

■- - - -■ HMM 80 $mgkg^{-1}day^{-1}$ (i.m.right leg)

×———× Control

Tumour implanted (i.m.) in left leg.

Treatment schedule days 1 - 5 : 13 - 17

Fig. 8.5 HMM against the M5 sarcoma

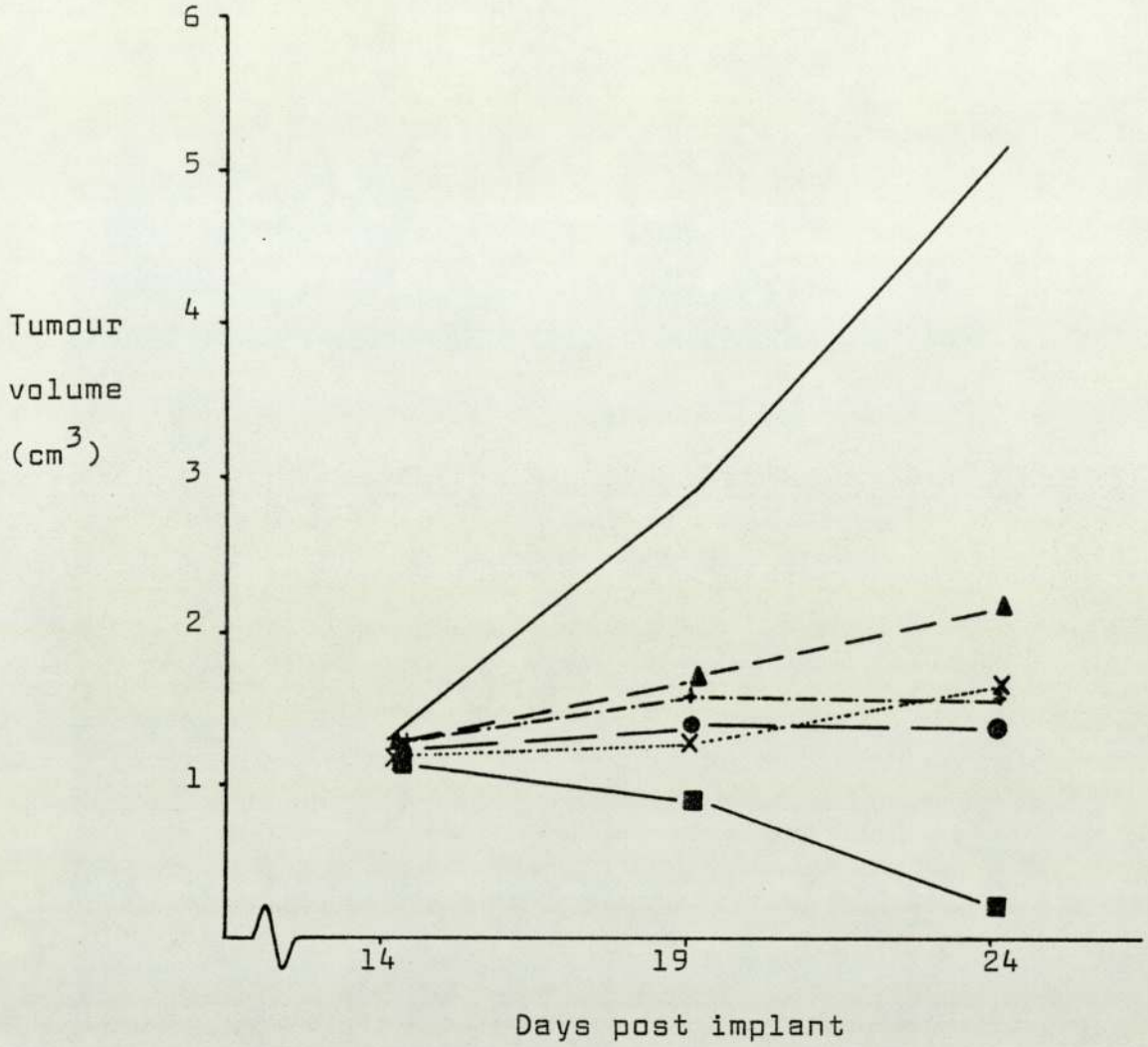


Fig. 8.6 Route dependence of HMM against the PC6 plasmacytoma

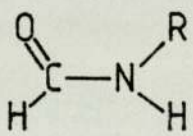
similar except for the i.p. route which was slightly superior. This route indepedence and apparent lack of direct acting ability of HMM would imply either that the drug is extremely bioavailable to the tumour site in the leg or more likely that the drug requires metabolism in some organ (probably the liver) thus making the distribution of cytotoxic species almost independent of the injection site.

In conclusion, therefore, formaldehyde clearly has the potential to kill tumour cells in vivo. However if formaldehyde is the final acting metabolite of HMM then it must either be generated at the tumour site (i.e. by metabolism) or brought to the tumour in some disguised form as a formaldehyde precursor.

Chapter 9 A structure-activity relationship study of N-methylformamide (NMF)

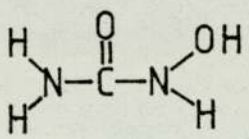
9.1 Introduction

Clinical trials with N-methylformamide (NMF; 13) are currently being conducted to ascertain whether this chemical solvent will find use as an antineoplastic agent. The apparent simplicity of the NMF molecule suggested that it might be an attractive model compound with which to determine a structure-activity relationship. The interpretation of its mode of action is uncomplicated by chemical instability (as is the case for molecules such as DTIC) and it was hoped that it might be suitably simple to unravel the N-methyl conundrum. Indeed formamide (F; 40) and NMF (13), alongside N-hydroxyurea (82) must surely represent the simplest organic molecules possessing antitumour activity if formaldehyde is excluded.



(13) R = CH_3

(40) R = H



(82)

The importance of the N-methyl group was investigated by varying the substituents on the nitrogen atom and examining the effect this had on the antitumour activity. The requirement of the formyl moiety was also examined by studying acetamide- and urea- type analogues.

Prior to investigating the structure-activity requirements of these molecules, the schedule dependency of NMF against the M5 sarcoma was assessed.

9.2 Schedule dependency of NMF

A total dose of 2000 mg kg^{-1} NMF was administered to BDF_1 mice implanted with the M5 sarcoma by a variety of different schedules. The results are given in table 9.1 and appendix 9.1 and three of these schedules are illustrated in fig. 9.1. The most effective schedules were those wherein the total dose was split into the longest chronic schedules e.g. A and D.

Schedule	Days of injection	Dose mg $kg^{-1}day^{-1}$	Total dose mg kg^{-1}	% Inhibition*
A	1 - 15	133	2000	82
B	1 - 10	200	2000	75
C	1 - 5 11 - 15	200	2000	76
D	1,3,5,7,9,11,13,15,17	220	2000	80
E	1,5,9,13,17	400	2000	75
F	1 - 5	400	2000	47
G	1,9,17	667	2000	60
H	1,9	1000	2000	52
I	1,17	1000	2000	56
J	1	1000	1000	35

* On day 25

Table 9.1 Schedule dependency of NMF against the M5 sarcoma

Intermittent high dose schedules e.g. H and I were clearly inferior and moreover produced greater weight losses. Thus

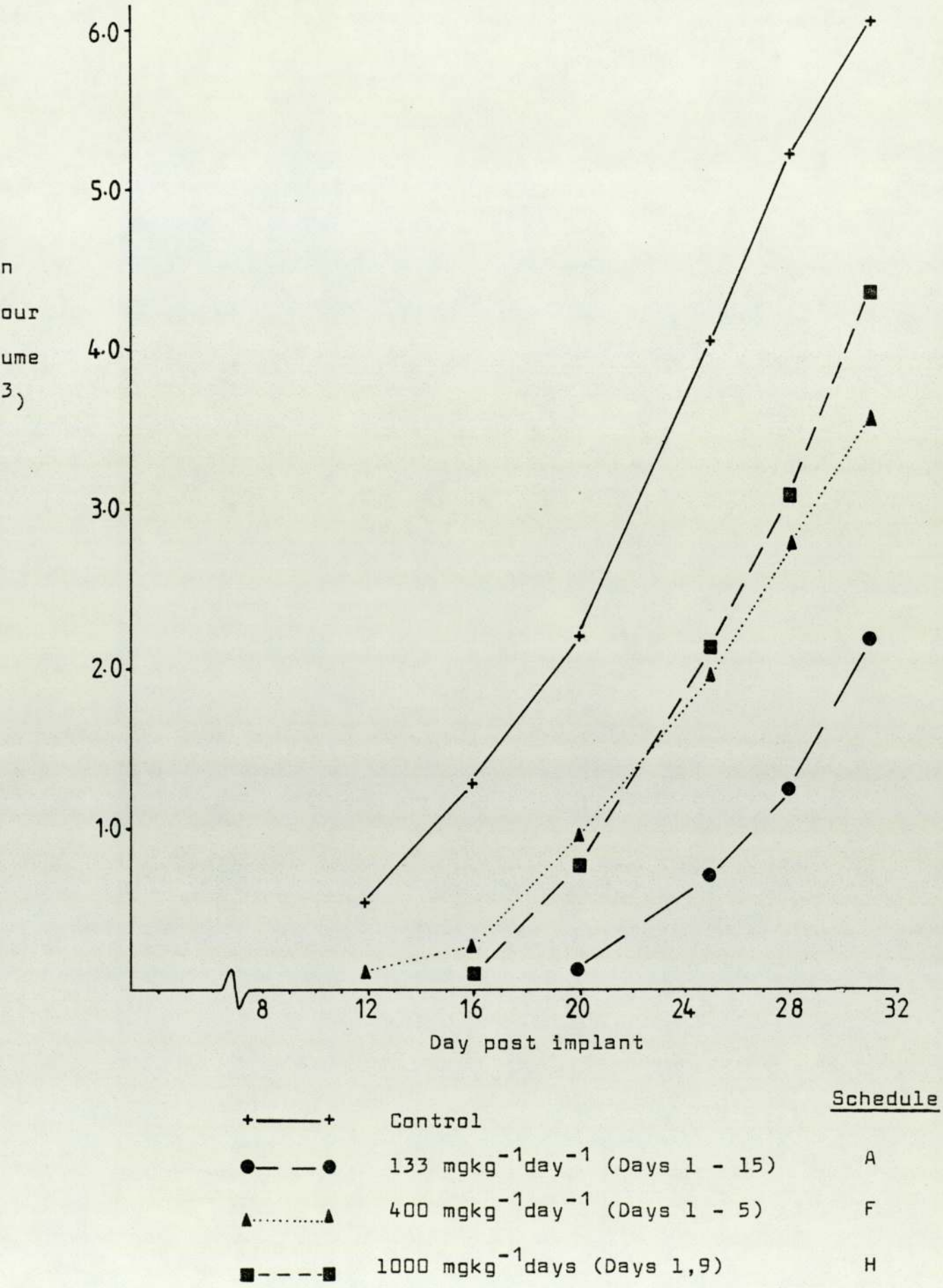


Fig. 9.1 Schedule dependency of NMF against the M5 sarcoma

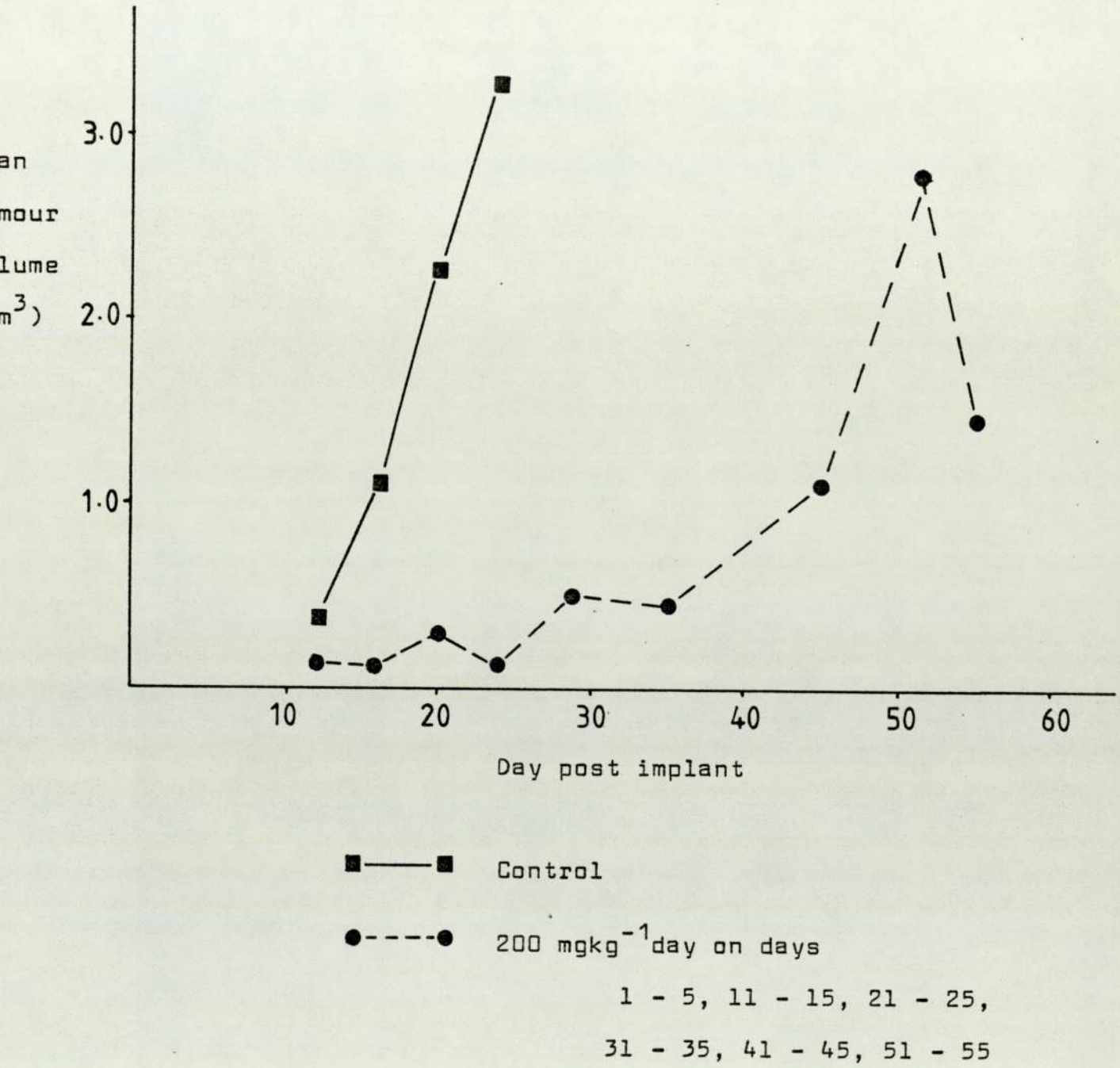


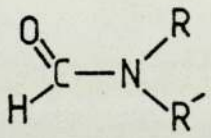
Fig. 9.2 NMF against the M5 sarcoma (On/off schedule)

a chronic daily schedule over 17 days was adopted for the general screening of the formamides.

NMF on a 5 day on/5 day off schedule over 60 days (fig.9.2 and appendix 9.2) produced long term tumour inhibition with no weight loss.

9.3 Studies of the activity of the N-substituted formamides against the M5 sarcoma and Sarcoma 180

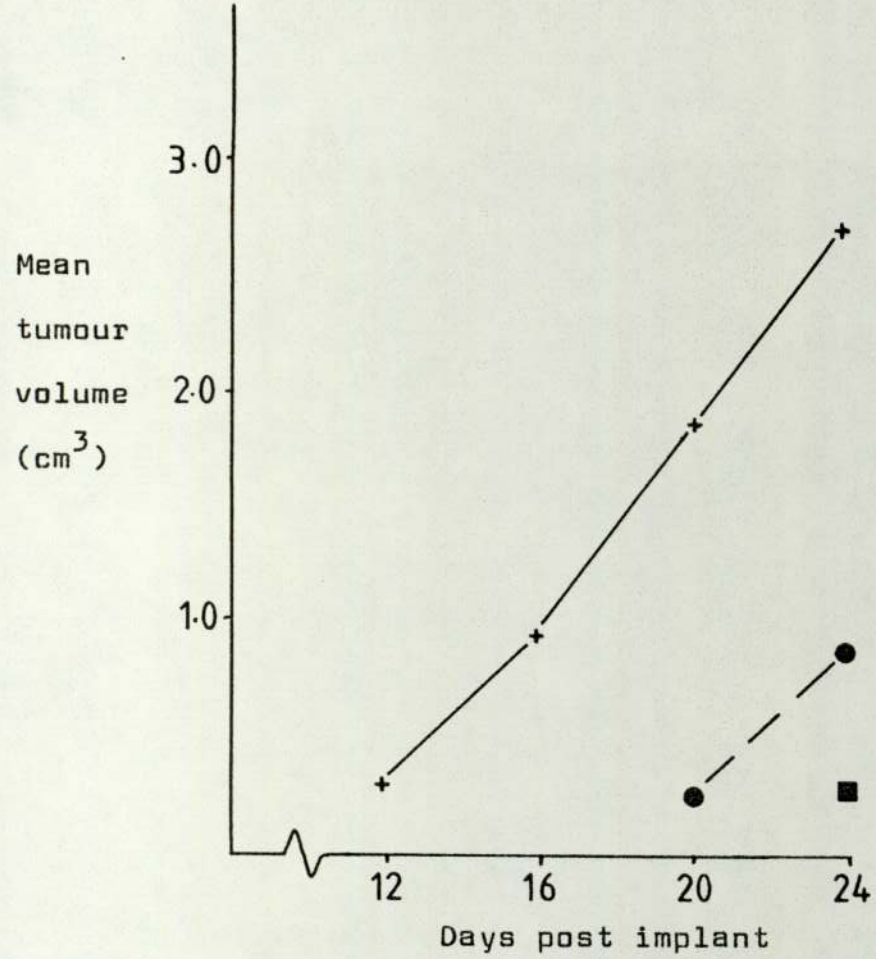
The results of a series of formamides tested against the M5 sarcoma are given in table 9.2, appendix 9.3 and illustrated in figs 9.3 and 9.4. In the N-alkylformamide series the only markedly active compound of those tested was NMF itself.



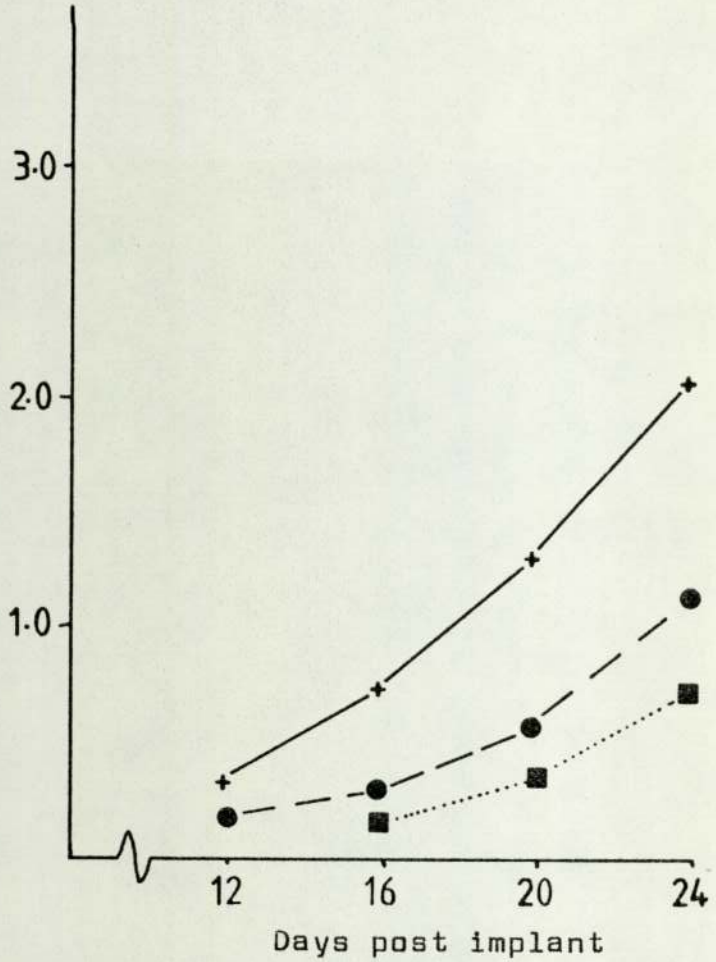
Compound No.	Name	R	R/	Schedule (days)	LD_{10} [a]	LD_{50} [a]	Optimal dose [a]	% Inhibition [b]
13	NMF	CH_3	H	1 - 17	220	300	200	100
40	F	H	H	1 - 17	200	270	200	63
41	DMF	CH_3	CH_3	1 - 17	1130	1280	1000	60
43	NEF	C_2H_5	H	1 - 17	320	420	300	22
83	HMF	CH_2OH	H	1 - 17	1580	1930	1500	63
84	HMMF	CH_2OH	CH_3	1 - 17	370	520	400	24

[b] = For optimal dose (day 24) [a] = mg $kg^{-1}day^{-1}$

Table 9.2 The activity of N-alkyl and N-hydroxymethylformamides against the M5 sarcoma

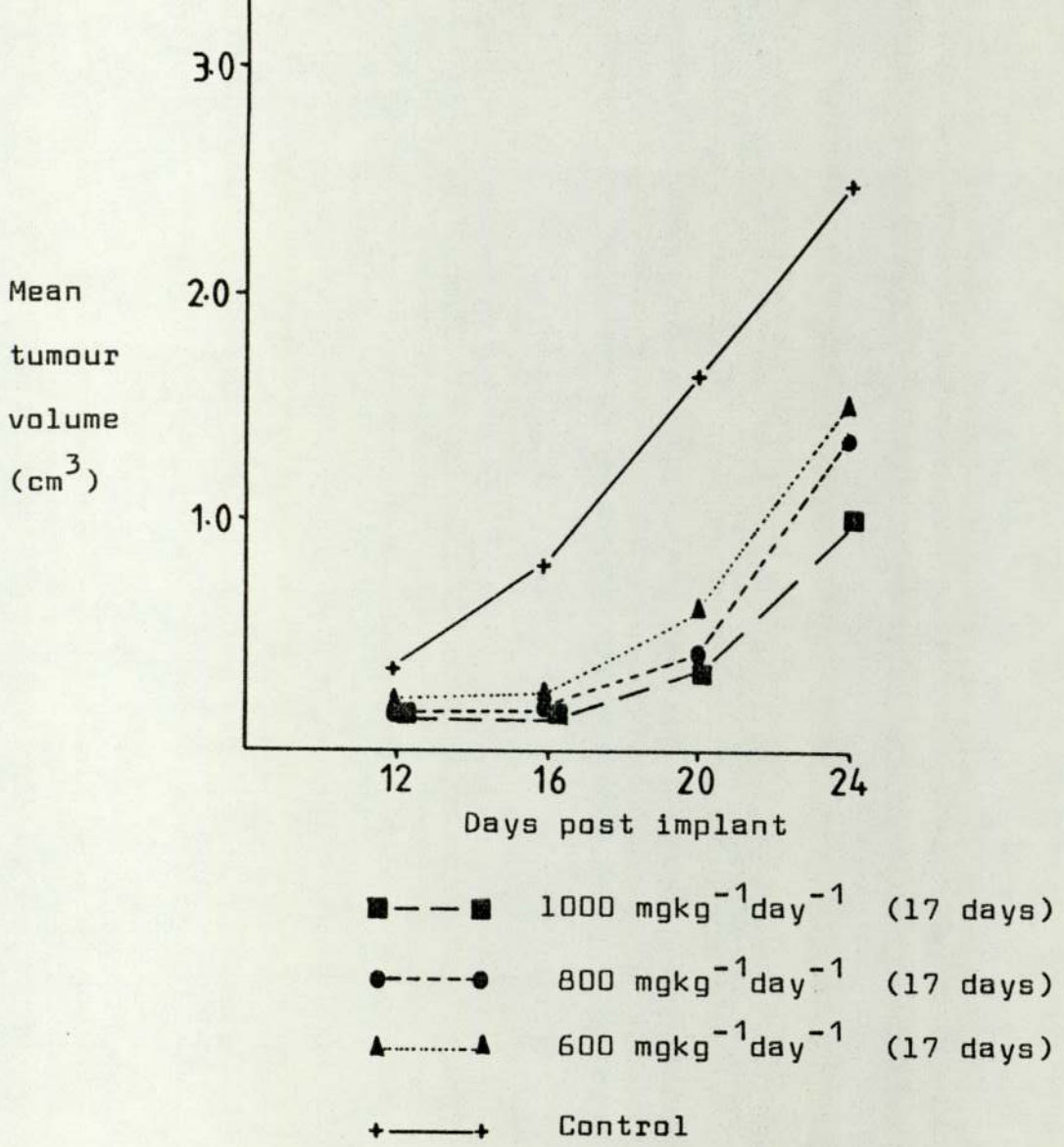


N-Methylformamide (NMF) against the M5 sarcoma

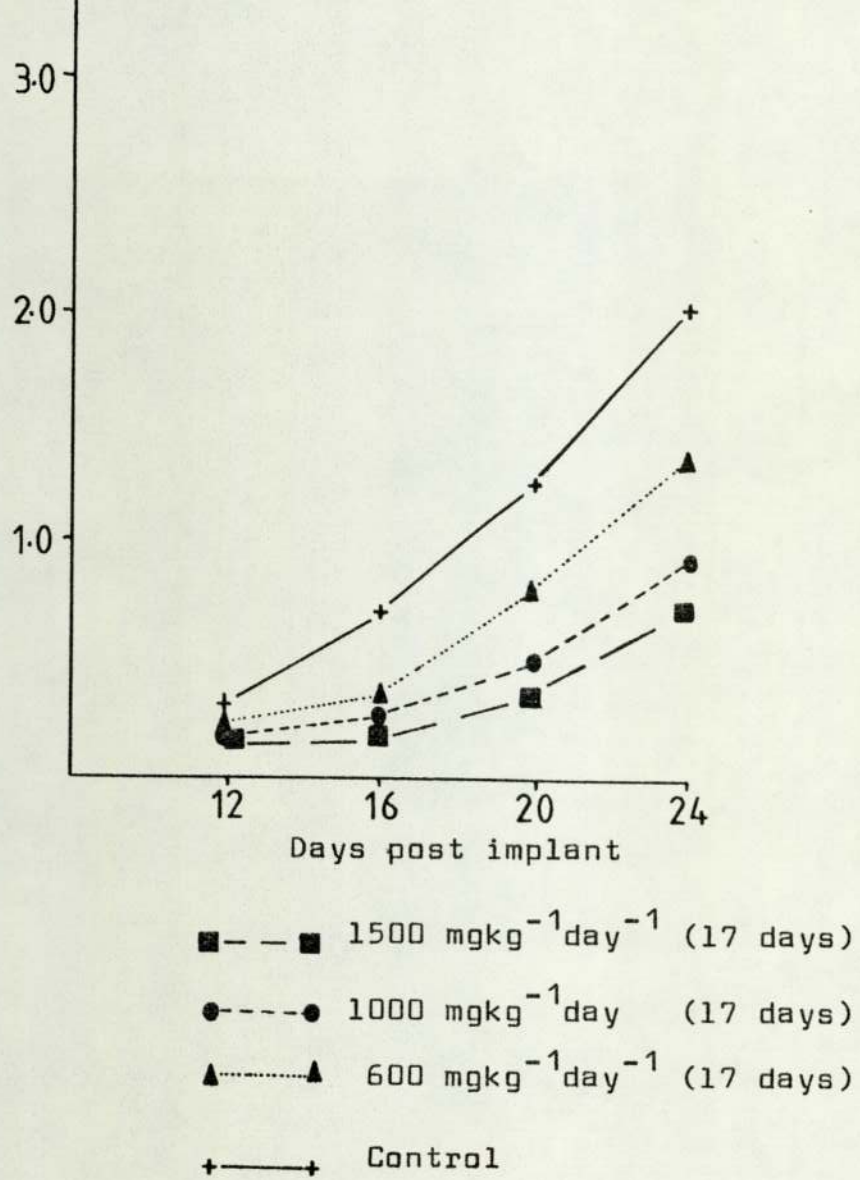


Formamide (F) against the M5 sarcoma

Fig. 9.3



NN-Dimethylformamide (DMF) against the M5 sarcoma



N-Hydroxymethylformamide (HMF) against M5 sarcoma

Fig. 9.4

F and NN- dimethylformamide (DMF; 41) were both capable of eliciting tumour inhibition whilst N-ethylformamide (NEF; 43) was completely devoid of antitumour activity. This inactivity of the N-ethyl analogue again parallels the findings for the ethyl analogues of HMM[98,106], the dimethyltriazenes[52] and procarbazine.[4,20] It is interesting that F retains activity, albeit low in comparison with NMF, since the desmethyl analogue of procarbazine,[4,27] and also melamine [98,106] are reported to be completely inactive against all tumours upon which they have been tested. However it should also be pointed out that whereas NMF demonstrates good activity against the TLX5 lymphoma, F is inactive against the same tumour.[119] DMF is thought to be metabolized to NMF and F[179,180] and production of the former *in vivo* may well account for the marginal activity obtained against the M5 tumour.

These N-alkylformamides were also tested against the Sarcoma 180 (table 9.3, fig. 9.5 and appendix 9.4). The results are in good agreement with those obtained by Clarke *et al*.[108] Overall the tumour is very much less sensitive to these compounds than is the M5 sarcoma, with both NMF and F producing a low degree of activity, whilst DMF and NEF are inactive. The difference between F and DMF is interesting since they are able to evoke similar optimal responses against the M5 sarcoma. One might speculate that if the activity of DMF is due to metabolic generation of NMF, then it is inactive against the Sarcoma 180, because NMF itself is only able to elicit marginal activity against this tumour.

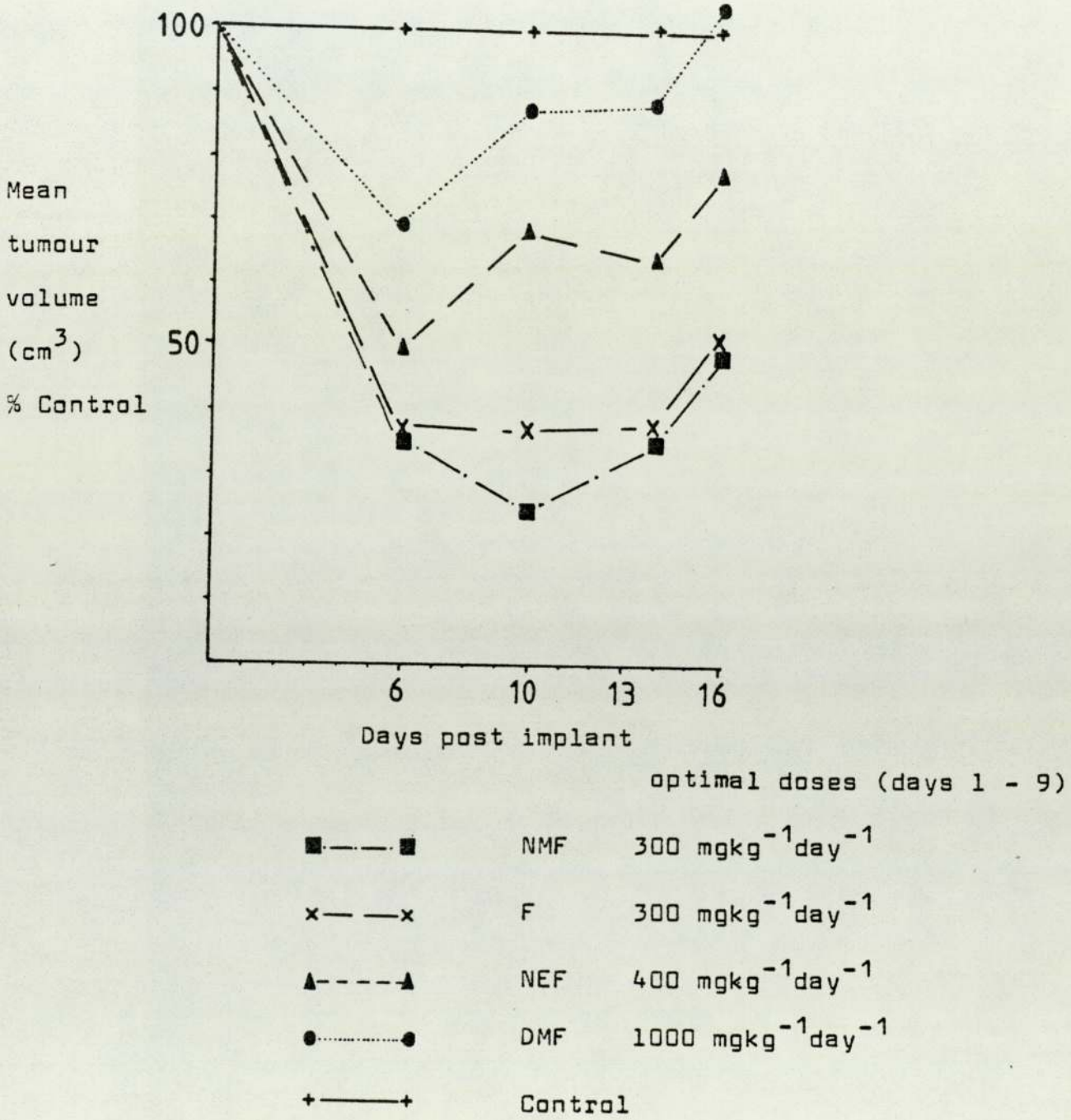
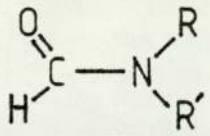


Fig. 9.5 Activity of the N-alkylformamides against the Sarcoma 180



Compound No.	Name	R	R/	Schedule (days)	LD_{10} $mgkg^{-1}$ day^{-1}	LD_{50} $mgkg^{-1}$ day^{-1}	Optimal dose $mgkg^{-1}$ day^{-1}	% * Inhibition
13	NMF	CH_3	H	1 - 9	313	370	300	52
40	F	H	H	1 - 9	320	400	300	47
43	NEF	C_2H_5	H	1 - 9	435	600	400	23
41	DMF	CH_3	CH_3	1 - 9	1040	1230	1000	0

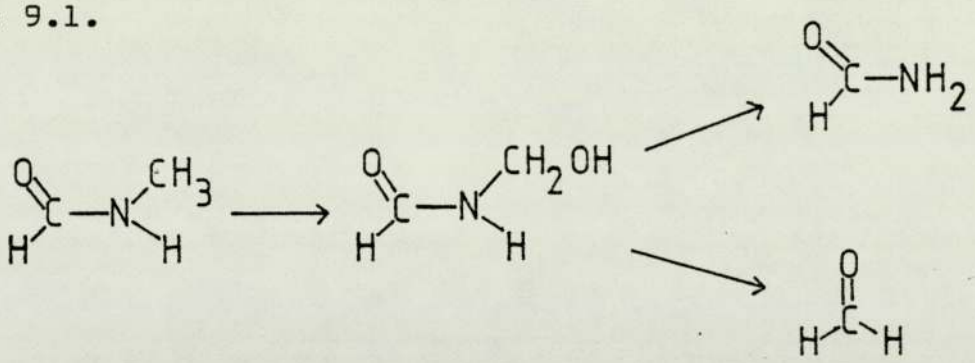
* For optimal dose (day 16)

Table 9.3 The activity of N-alkylformamides against the Sarcoma 180

Gescher et al.[119] have investigated the effects of both NMF and NEF against TLX5 lymphoma cells in vitro and shown them to be equally cytotoxic. In vivo NMF demonstrates marked activity against TLX5 cells whereas NEF is inactive.[119] This parallels the finding that monomethyl- and monoethyltriazenes are equally cytotoxic towards these cells and yet only the methyl compound is an antitumour agent in vivo.[38] Both NMF and NEF are metabolized to F in vivo. However attempts to metabolize NMF in vitro by liver microsomes or by isolated hepatocytes have not yielded detectable levels of metabolites.[119] NMF has also been shown to deplete hepatic non-protein thiols in vivo, an effect not

produced by NEF nor F.[119] This depletion may well be associated with the hepatoxicity and antitumour activity of the drug.

Since a compound identified as F on g.c. analysis has been found in the urine of mice treated with NMF, it is clear that NMF does undergo metabolism.[119] A urinary metabolite has also been identified as a stable precursor of formaldehyde.[119] This suggests oxidative metabolism via a carbinolamide as in scheme 9.1.



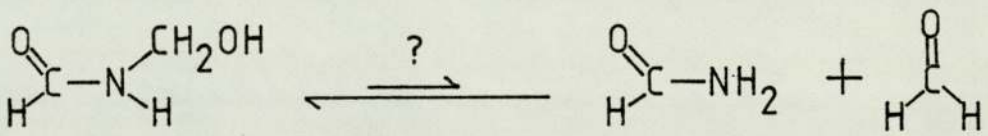
Scheme 9.1 Proposed metabolism of NMF

The carbinolamide, N-hydroxymethylformamide (HMF; 83) was tested against the M5 sarcoma and demonstrated a degree of activity comparable with that of F and DMF. It was however markedly less toxic than either NMF or F <u>in vivo</u> as demonstrated by the LD_{10} and LD_{50} values (table 9.2).

<u>In vitro</u>, HMF (30mM) is reported to produce an approximately 4 log cell kill of TLX5 lymphoma cells when incubated for 2h at 37°C whereas NMF (<600mM) was without toxicity.[127]

Similar results have also been obtained using human ovarian carcinoma cells.[127] These results present a considerable paradox when considered against the *in vivo* data.

When HMF is dissolved in aqueous solution and assayed by the method of Nash,[177] it is found to be contaminated with small amounts of formaldehyde.[176] It is unknown whether this formaldehyde arises from inefficient purification of the compound (since paraformaldehyde is a starting material in its synthesis) or whether HMF decomposes in aqueous solution to release formaldehyde (scheme 9.2).



Scheme 9.2 Possible decomposition of HMF

Thus in incubations of the compound, the carbinolamide is not being examined solely *per se* since this highly cytotoxic contaminant is present. It is therefore difficult to make absolute statements concerning its cytotoxicity.

HMF inhibits the incorporation of radiolabelled formate leucine, uridine and thymidine into the cellular macromolecules of TLX5 cells. The inhibition of uridine incorporation can be prevented by preincubation of the cells with semicarbazide, a result taken to imply that the inhibition is caused by formaldehyde.[127] However it is also possible that semicarbazide may react directly with HMF.

A simple *in vivo* test was conducted against the ascitic (i.p.) M5 sarcoma (table 9.4). HMF was compared with NMF and also

Compound	Dose $mg.kg^{-1}$ day^{-1}	Schedule	Mean death day ($\pm$ s.d.)	$\frac{T}{C}$ X 100%	% * I.S.T
NMF	250	1 - 9	33.6 $\pm$ 4.8	131	31
HMF	318	1 - 9	27.0 $\pm$ 3.5	105	5
F	19	1 - 9	27.1 $\pm$ 3.1	106	6
Formaldehyde	13	1 - 9	31.6 $\pm$ 3.6	123	23
Control			25.6 $\pm$ 3.2	100	

* I.S.T. = Increase in survival time of treated over control mice

Table 9.4 NMF and potential metabolites against the ascitic M5 sarcoma

with F and formaldehyde. Equimolar amounts of NMF and HMF were employed and the amounts of F and formaldehyde were 10% of these values. These last two were present to act mainly as controls for potential contaminants of HMF but they also represent metabolites of NMF. If HMF is indeed the active metabolite of HMF and is as cytotoxic as suggested by the *in vitro* incubations, then when injected i.p. directly into the same compartment as the tumour cells one might expect a greater cell kill than for NMF. Within this system NMF has the potential to be metabolized to its active form (if necessary), however if HMF were the active metabolite it is unlikely that levels as high could be achieved as might by direct injection of HMF. Although NMF barely produced significant activity against the ascitic form of this tumour,

it is clear that HMF was completely without activity and certainly not a highly cytotoxic species.

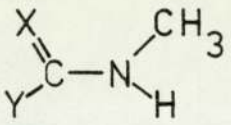
Another i.p./i.p. tumour system against which both NMF and HMF have been tested is the i.p. P388 leukaemia. NMF (optimal I.S.T. = 54%) again demonstrated a superior response to HMF (optimal I.S.T. = 27%).[181]

It thus appears that HMF is unlikely to be the active metabolite of NMF although it does appear to be formed *in vivo*. Cooksey *et al*. have suggested that its formation may represent a deactivation pathway of NMF metabolism.[127]

N-Hydroxy-N-methylformamide (HMMF; 84) is a proposed intermediate of the metabolism of DMF to NMF. It is rather more toxic than HMF yet is completely without activity against the M5 sarcoma (table 9.2). Interestingly this compound is also extremly cytotoxic[130] to human ovarian cells but again formaldehyde is present as a contaminant.

9.4. Studies of N-methyl substituted acetamides and ureas against the M5 sarcoma

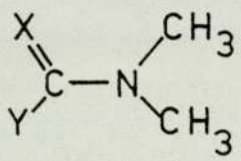
A series of derivatives of acetamide and urea containing either a monomethylamino (table 9.5 and appendix 9.5) or a dimethylamino group (table 9.6 and appendix 9.5) were screened against the M5 sarcoma. All of these compounds were found to be inactive which might be interpreted as indicating the importance of the formyl group.



X	Y	Schedule (days)	LD_{10} *	LD_{50}*	Optimal dose *	% Inhibition ‡
O	CH_3-	1 - 17	800	1240	800	0
O	CF_3-	1 - 17	400-800	-	400	3
O	CH_3NH-	1 - 17	1730	2260	1600	34
S	$(CH_3)_2N$-	1 - 17	450	720	400	9

* - $mgkg^{-1}day^{-1}$ ‡ - day 24

Table 9.5 Activity of urea and acetamide derivatives containing a monomethylamino moiety against the M5 sarcoma.

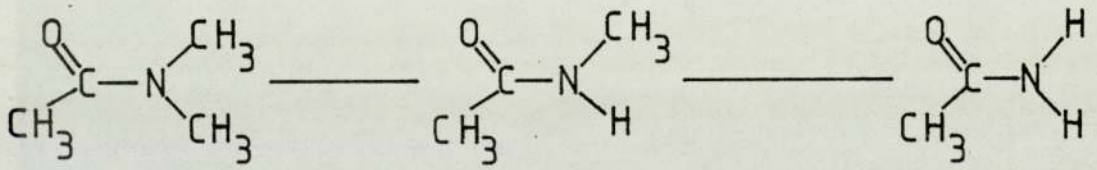


X	Y	Schedule (days)	LD_{10} *	LD_{50}*	Optimal dose *	% Inhibition ‡
O	CH_3-	1 - 17	900	1420	800	22
O	$(CH_3)_2N$-	1 - 17	440	640	400	33
S	H-	1 - 17	185	260	200	3
S	$(CH_3)_2N$-	1 - 17	430	565	400	21
NH	$(CH_3)_2N$-	1 - 17	107	142	100	0

* $mgkg^{-1}day^{-1}$ ‡ - day 24

Table 9.6 Activity of urea and acetamide derivatives containing a dimethylamino moiety against the M5 sarcoma.

It was thought that molecules such as N-methylacetamide (85) and N-methyl-trifluoroacetamide (86) might be so similar in size to NMF that they could retain some antitumour activity. Possibly unfavourable physicochemical properties or electronic factors militate against this hypothesis. NN-Dialkylacetamides are known to produce a small degree of inhibition against the adenocarcinoma 755[182] tumour and on the basis of this NN-dimethylacetamide (89) was selected for a phase 1 clinical trial.[183] CNS toxicity with hallucinations induced by the drug led to its withdrawal.[173] It is known to be metabolized to monomethylacetamide (85) and acetamide <u>in vivo</u> presumably following a pathway (scheme 9.3) analogous to the metabolism of DMF.[179] It is, however, quite inactive



(89) (85)

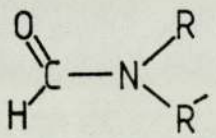
Scheme 9.3 Metabolism of NN-dimethylacetamide

towards the M5 sarcoma.

Two observations suggested that the N-methyl substituted urea structures might be of interest. Firstly NNN′N′-tetramethylurea (90) is active against the YPC-1 plasma cell tumour.[184] In addition the compound undergoes N-demethylation to produce NNN′-trimethylurea.[185] Secondly links exist between the methylated ureas, formamides and acetamides in

their abilities to induce differentiation of Friend leukaemia cells *in vitro*,[186] to be teratogenic to rats and rabbits[187] and to cryopreserve erythrocytes.[186] It is unknown whether any link exists between these disparate biological properties and antitumour activity. Both the N-methyl substituted ureas (87,90) were inactive as were the substituted thioureas (88,92) and tetramethylguanidine (93), the last proving to be comparatively toxic.

In conclusion, considering the inactivity of the above acetamide and urea type compounds, it would appear that the formyl moiety is one vital component for antitumour activity within the formamide molecule and that the alkyl substituents (R and R′) on the nitrogen atom might provide fine tuning of biological activity, optimal activity being achieved with NMF.



Basic structure for activity against the M5 sarcoma.

Chapter 10 The Toxicology of N-methylformamide (NMF)

10.1 Introduction

As NMF had demonstrated convincing activity against several human tumour xenografts implanted in mice and also against several murine tumour models,[108,119], a clinical trial of the drug was initiated. As part of the preclinical investigations optimal dosing of the drug was determined and a detailed study of several aspects of its toxicity investigated.

10.2 Lethal doses (LD_{10} and LD_{50} values)

Retrospective analyses of drugs which have been tested in humans suggest that mouse toxicological data alone without subsequent large animal toxicology is sufficient to predict useful starting doses of anti-cancer drugs in humans.[188] Ideally the maximum tolerated dose i.e. 'the highest dose consistent with tolerable and reversible side effects at a given schedule in a specific population of patients' is approached in the clinical trial as rapidly as possible and this can be predicted approximately from a knowledge of the mouse LD_{10} value.[188] In these experiments the choice of routes and schedules of administration was based on the C.R.C. phase 1 clinical trials requirements.[130] The Balb/c mouse was selected as the murine strain since this was considered to be a comparatively weak species thus predicting lower and therefore safer lethal values. Balb/c mice are now known to be more susceptible to the toxicity of NMF than either the CBA/ca or BDF_1 strains.

The acute LD_{10} and LD_{50} values were determined for the intraperitoneal (i.p.), per os (p.o.), intramuscular (i.m.) and intravenous (i.v.) injection routes. The multiple dose LD_{10} and LD_{50} values were also determined for a course of 5 successive daily treatments. The results are tabulated in table 10.1

Route	Schedule	LD_{10} (mg $kg^{-1}day^{-1}$)	LD_{50}(mg $kg^{-1}day^{-1}$)
i.p.	day 1 only	800	2300
p.o.	day 1 only	2000	2700
i.v.	day 1 only	900	1480
i.m.	day 1 only	2000	2700
i.p.	days 1 - 5	380	490

Table 10.1 The lethal dose values of NMF in Balb/c mice.

Except for those mice which had received NMF by the i.v. route, the mice died after a period of several days during which time they progressively lost weight and ceased to groom themselves. After i.v. injection of a lethal dose of NMF, death occurred consistently within 5 minutes.

If one compares the LD_{10} values of NMF with those of some of

Agent	LD_{10} value (mol $kg^{-1}day^{-1}$; i.p.)	
	Days 1 - 17[a]	Day 1 only[b]
NMF	2.7×10^{-2}	1.4×10^{-2}
Methotrexate	5.7×10^{-6}	-
Adriamycin	6.4×10^{-6}	-
Cyclophosphamide	-	1.3×10^{-3}

a - BDF_1 mice b - Balb/c mice

Table 10.2 The lethal dose values of NMF relative to other antineoplastic agents.

the clinically employed antineoplastic agents (table 10.2) on the basis of moles kg^{-1} of drug given, then it is clear that NMF is an agent of relatively low toxicity.

10.3 Haematological toxicity - Introduction

The majority of the currently employed antineoplastic drugs produce marked bone marrow suppression. This leads to leukopenia and thrombocytopenia which in turn increases the animal's susceptibility to infection and can allow internal bleeding to occur unabated. Therefore these manifestations of toxicity have to be carefully monitored in patients receiving anticancer drugs and for many haematological toxicity proves to be dose-limiting.

In the early clinical report of the hepatotoxicity induced by NMF, no haematological toxicity was observed in the patients monitored.[118] Anglesio et al., in a study of certain toxic effects of antitumour agents in rats, determined the white blood cell counts after a single chronic schedule of NMF (150 mg $kg^{-1}day^{-1}$ for 5 days).[189] No difference in the count was observed between the first and the last days of treatment. Rather disconcertingly however a similar result was obtained with methotrexate.

Absence of haematological toxicity is a very desirable attribute of an antitumour agent. If, during a course of treatment, therapy is delayed until complete marrow recovery has taken place then tumour cells will be allowed to proliferate unchecked.

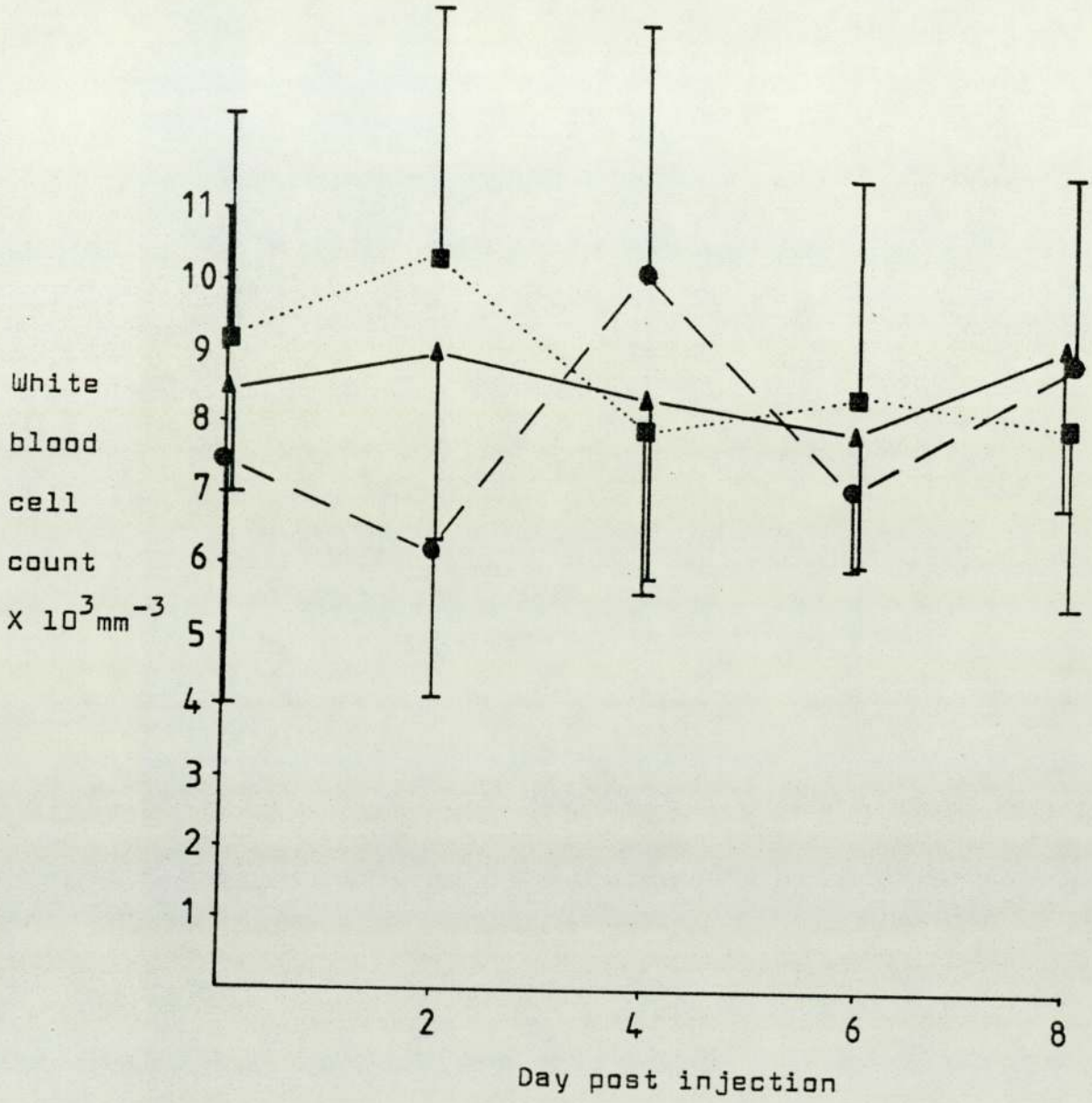
A non-marrow toxic agent might usefully be employed at this stage. Therefore since this toxicity, or lack of it, is of considerable importance to the potential use of the drug in the clinic, investigations were conducted into the effects of NMF on the levels of peripheral blood cells.

10.4 Peripheral blood counts in mice after treatment with NMF

White blood cell, erythrocyte and platelet counts were monitored after injection of large single doses of NMF. The acute LD_{10} (800 mg kg^{-1}; i.p.) and LD_{50} (2300 mg kg^{-1}; i.p.) doses were chosen since any change if present should be very obvious at these levels. Cyclophosphamide and CCNU at their acute LD_{10} values (320 mg kg^{-1} and 40 mg kg^{-1} respectively) served as positive controls. Solvent controls were also used.

The white blood cell counts are tabulated in appendix 10.1 and illustrated in figs 10.1.a and 10.1.b. Whereas both cyclophosphamide- and CCNU- treated mice demonstrated pronounced leukopenias with nadirs of peripheral white cells at about day 4 after injection, NMF produced only a slight depression at its LD_{50} value which returned to normal by day 4. At the LD_{10} dose no change from the control value was seen suggesting that the drug produced no significant leukopenia.

Figs 10.2.a and 10.2.b (appendix 10.2) show the platelet counts for NMF and again no fall was seen. Both CCNU- and cyclophosphamide- treated mice showed a small but significant change compared with the control values (fig. 10.2.b).

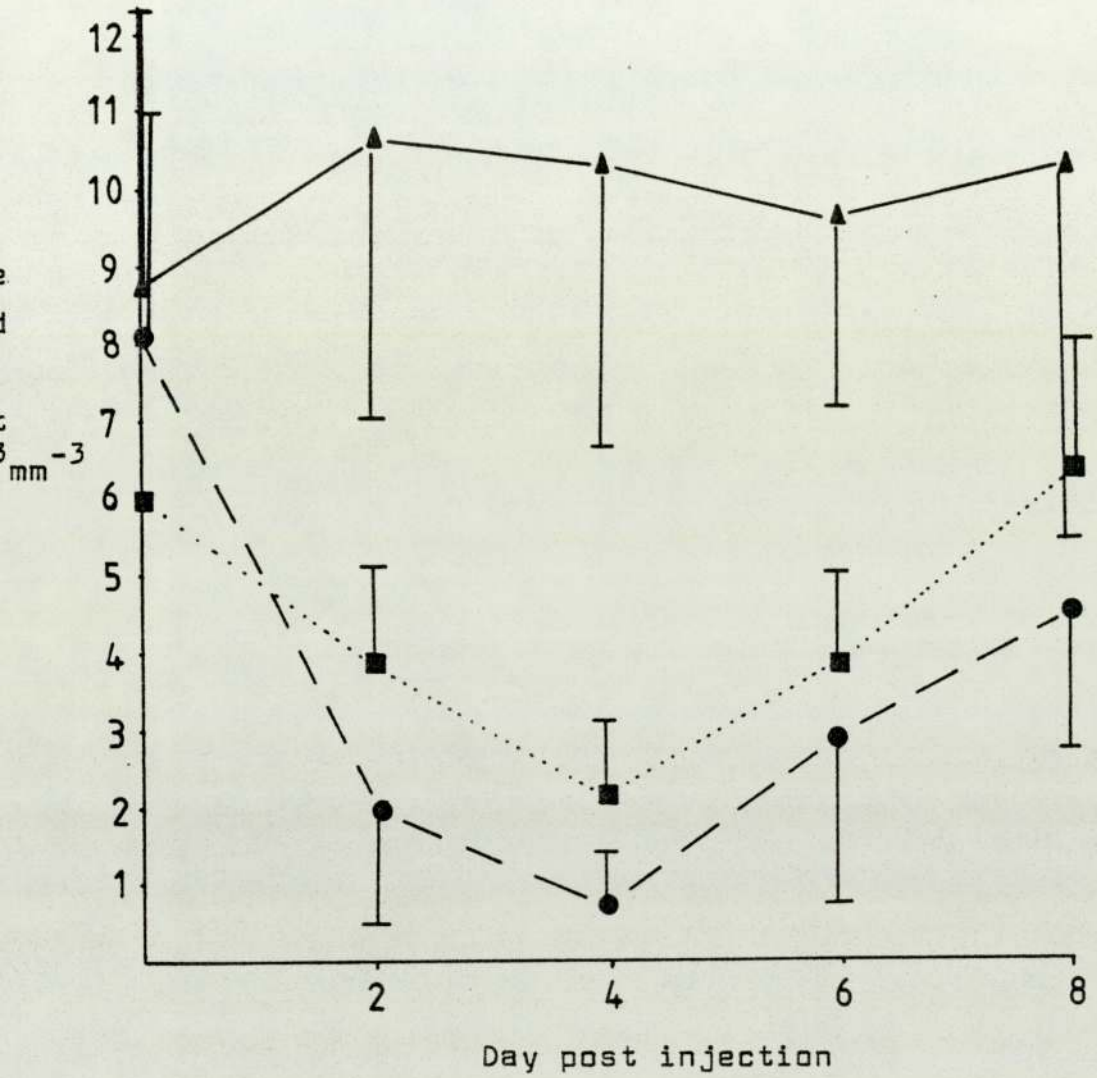


●— — —● NMF 2300 $mgkg^{-1}$

■·····■ NMF 800 $mgkg^{-1}$

▲——▲ Control (saline)

Fig. 10.1(a) Peripheral white blood cell counts (± s.d.) of mice (balb/c) treated with a single injection of NMF



●– – –● Cyclophosphamide 320mgkg^{-1}

■·····■ CCNU 40 mgkg^{-1}

▲——▲ Control (10% DMSO/Oil)

Fig. 10.1(b) Peripheral white blood cell count (± s.d.) of mice (balb/c) treated with cyclophosphamide or CCNU

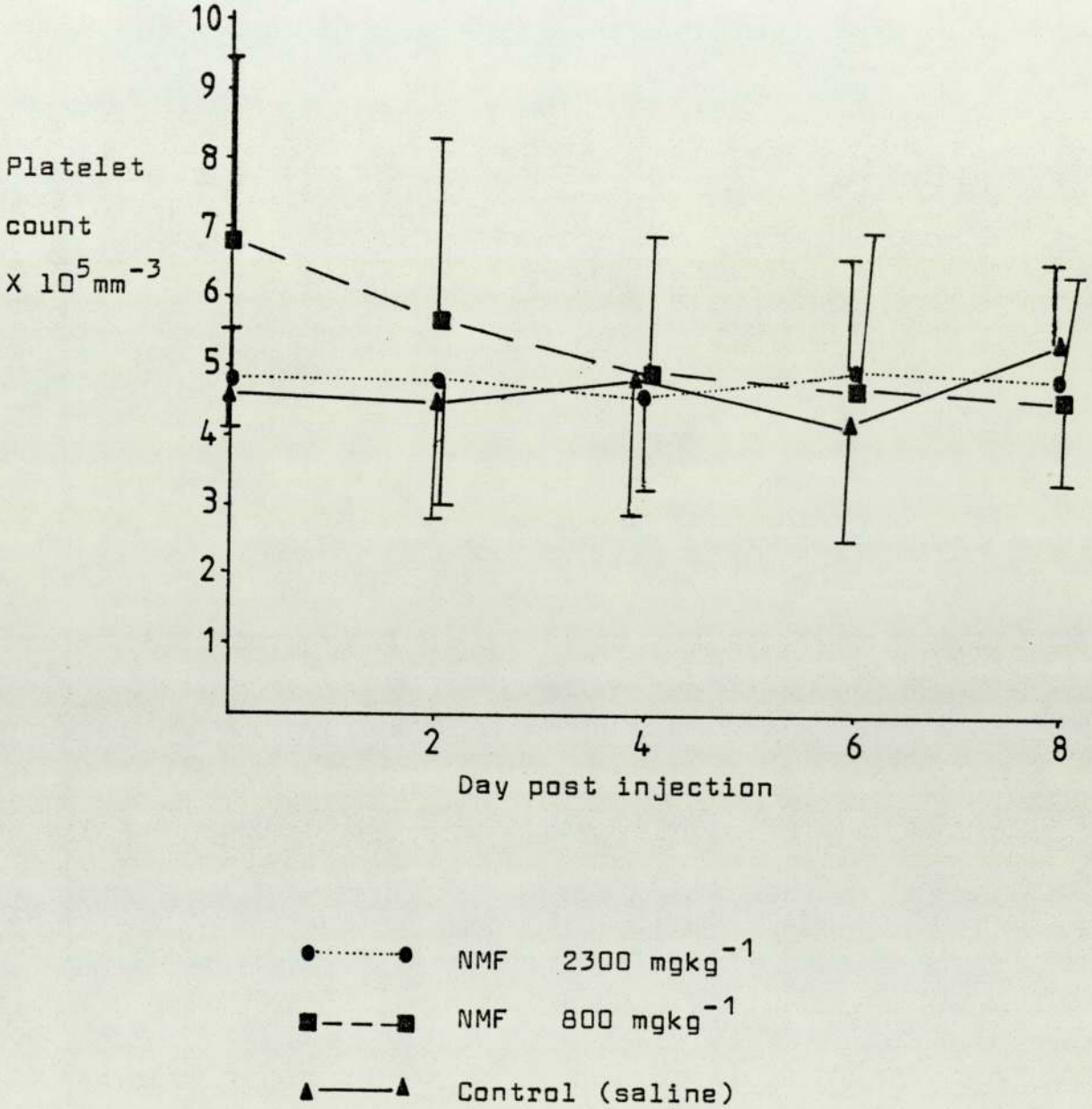


Fig. 10.2(a) Peripheral platelet counts (± s.d.) of mice (balb/c) treated with a single injection of NMF.

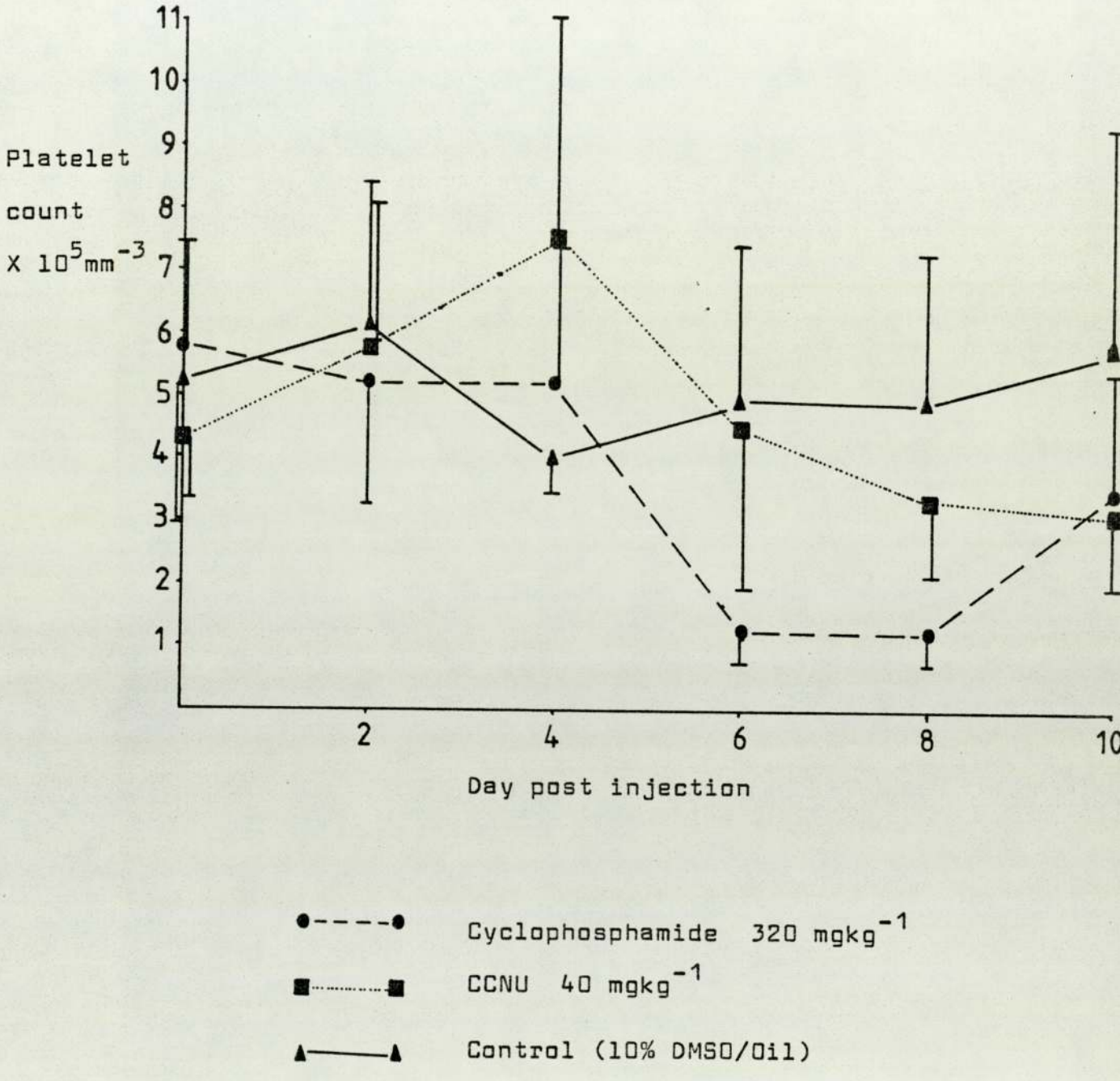


Fig. 10.2(b) Peripheral platelet counts ($\pm$ s.d.) of mice (balb/c) treated with cyclophosphamide or CCNU.

None of these agents produced any significant change in the erythrocyte counts. These are given in appendix 10.3.

To ensure that there were no changes over a longer period, the blood elements were monitored over a 4-week time course. Chronic schedules of NMF dosing were also investigated. As seen in appendices 10.4 - 10.6 no significant changes other than those demonstrated for the positive controls within the first week were observed.

These results indicate that NMF is not bone marrow toxic in this strain of mice even at lethal dose levels as manifested by normal peripheral blood counts. Preliminary results from current clinical trials with the drug confirm that this finding extends to humans.[190] This lack of bone marrow toxicity is of great relevance to the potential use of the drug. If the drug is found to be of use in the treatment of a particular tumour then it might be added, with effect, to a combination which possesses myelosuppression as the dose limiting toxicity. The proviso is that the other drugs would have to lack hepatotoxic potential and also that the hepatotoxicity of NMF would not interfere in a detrimental manner with their metabolism and consequent effectiveness. Alternatively the drug might find use in patients who have pre-existing abnormal bone marrow function as is often the case after prior chemotherapy or radiotherapy.

10.5 Hepatotoxicity of NMF in mice

The first clinical trial of NMF in man drew the conclusion

that hepatotoxicity might prove to be the dose limiting toxicity for this agent[118]. Hepatotoxicity has been demonstrated also in both rats[191,192] and dogs[193] treated with NMF. Since the potential of the drug as a constituent of combination chemotherapy was to be examined it was of interest to know at which doses NMF demonstrated hepatotoxicity in mice.

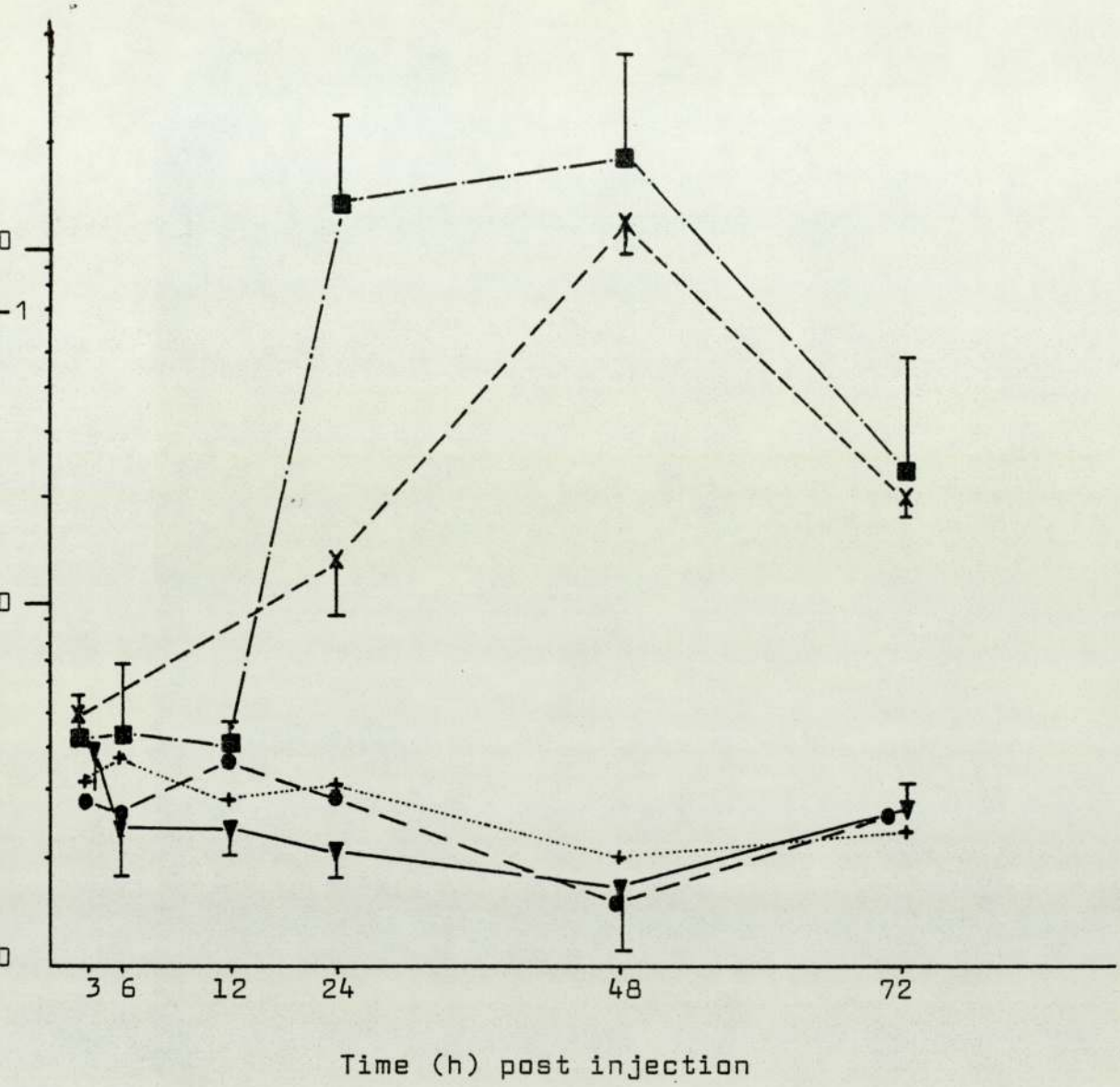
There are many methods available for the evaluation of liver function. The measurement of levels of specific enzymes in the plasma or serum are the most widely performed liver function tests. The levels of three enzymes, sorbitol dehydrogenase (SDH), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were studied for the following reasons. SDH is an example of an enzyme with a high degree of organ specificity; significant quantities of the enzyme are found only in the liver, whereas it is barely detectable in normal plasma and other organs.[194] Thus an elevation in the plasma may be taken to be indicative of liver damage. Secondly Lundberg et al. have recently demonstrated a correlation between elevated serum levels of SDH and histopathological damage in rat livers after treatment with DMF and NMF[191]. ALT and AST were chosen since they are two of the most widely used liver function markers and levels of both have been shown to be increased in the sera of man and dog after treatment with NMF.[190,193]

Female BDF_1 mice were administered NMF over either a chronic 10 day schedule or as a single acute injection. Plasma

enzyme levels were monitored at various time points after administration of the drug and the results are illustrated in figs 10.3 - 10.6. (appendices 10.7 - 10.10).

Levels of SDH after a single injection of NMF are depicted in fig 10.3. At doses of 400 mg kg^{-1} or less no elevation of this enzyme was observed while at doses of 800 and 1200 mg kg^{-1} marked increases in levels were observed after 24h. Maximum levels were achieved between 24 and 48h post injection. After a chronic 10 day schedule SDH levels were monitored at various time points after the final injection (fig 10.4). Elevated levels were observed at 400 mg kg^{-1} but not at 200 mg kg^{-1}. ALT and AST levels after a chronic 10 day schedule are illustrated in figs 10.5 and 10.6. At 400 mg $kg^{-1}day^{-1}$ ALT levels rose in parallel with the SDH levels whilst AST levels were only slightly raised. At 200 mg $kg^{-1}day^{-1}$ both ALT and AST levels were identical to control values.

Balb/c mice (acute LD_{10}= 800 mg kg^{-1}) are markedly more susceptible than BDF_1 mice (acute LD_{10} = 2200 mg day^{-1}) to the lethal effects of NMF. While Balb/c mice demonstrate normal plasma levels of enzymes at 24h after injection of 100 mg kg^{-1} NMF, at 150 mg kg^{-1} elevated levels of SDH (199-fold), ALT (166-fold) and AST (37-fold) were found. Thus it is feasible that Balb/c mice are more susceptible due to the onset of hepatotoxicity at lower doses in this strain.



X-----X 1200 mg kg^{-1} NMF

■-—-—■ 800

+·····+ 400

●----● 200

▼——▼ Saline control

Fig 10.3 Plasma levels of sorbitol dehydrogenase (SDH) in female BDF_1 mice after a single injection of NMF.

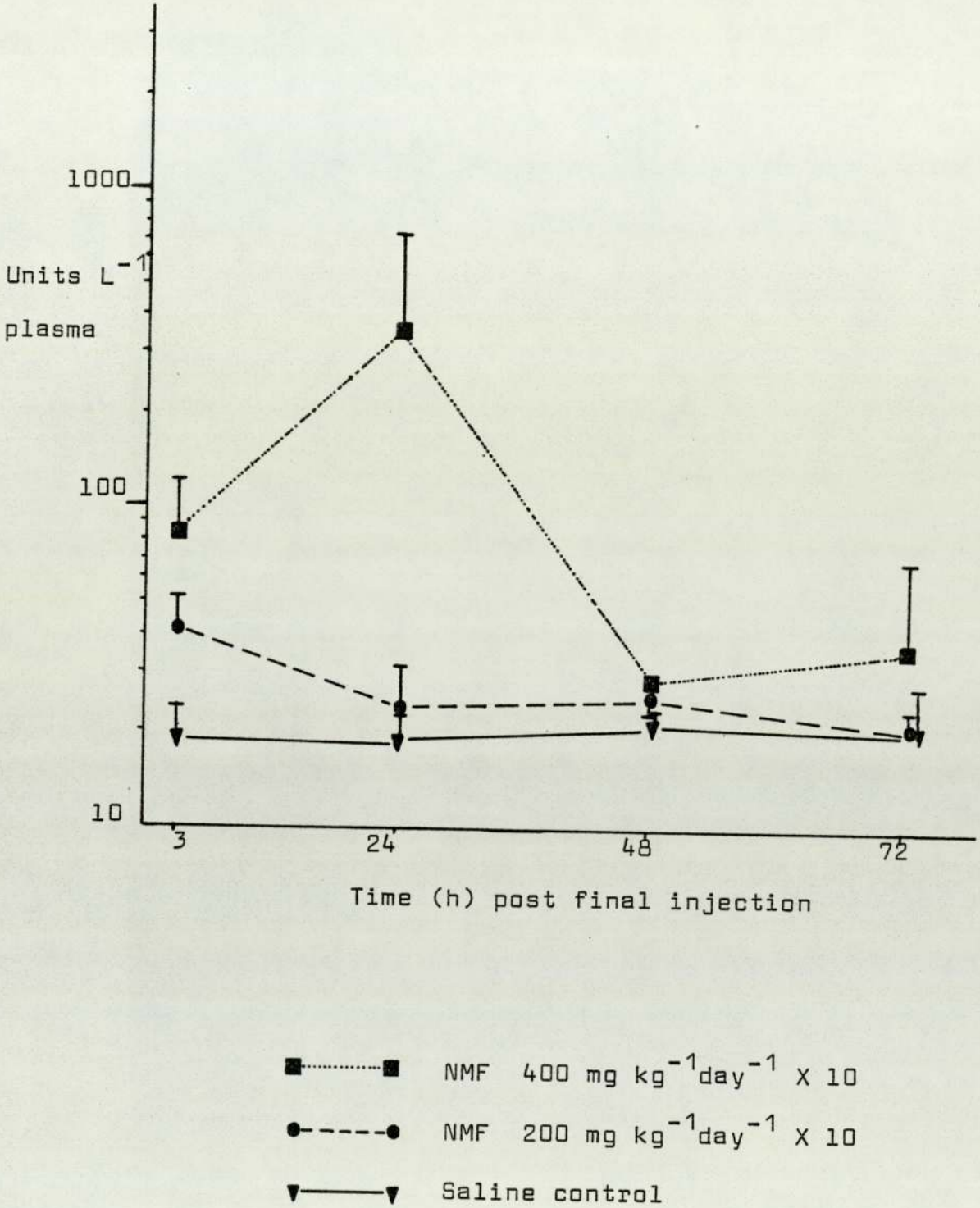
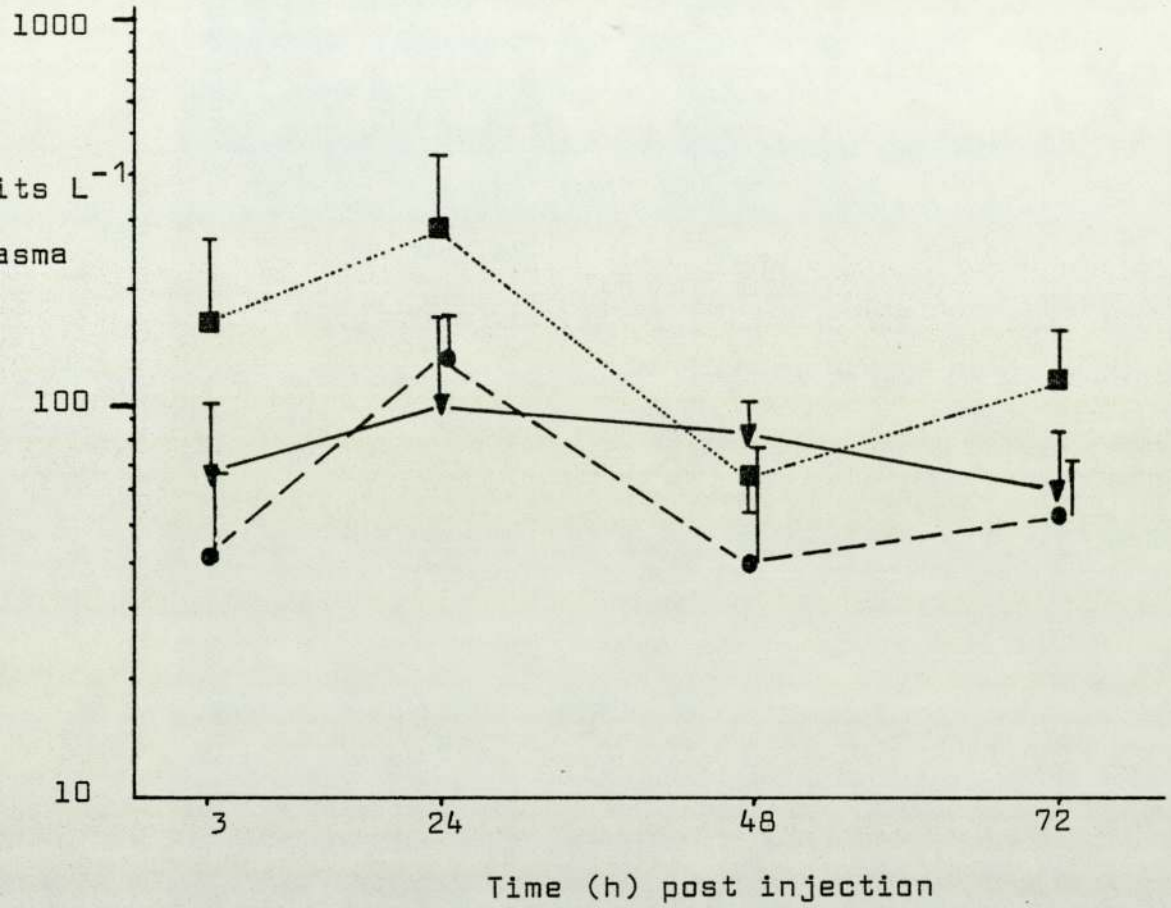


Fig 10.4 Plasma levels of sorbitol dehydrogenase (SDH) in female BDF_1 mice after a chronic (10 day) schedule of NMF.



■·····■ NMF 400 mg $kg^{-1}day^{-1}$ X 10

●– – –● NMF 200 mg $kg^{-1}day^{-1}$ X 10

▼——▼ Saline control

Fig 10.5 Plasma levels of aspartate aminotransferase (AST) in female BDF_1 mice after a chronic (10 day) schedule of NMF

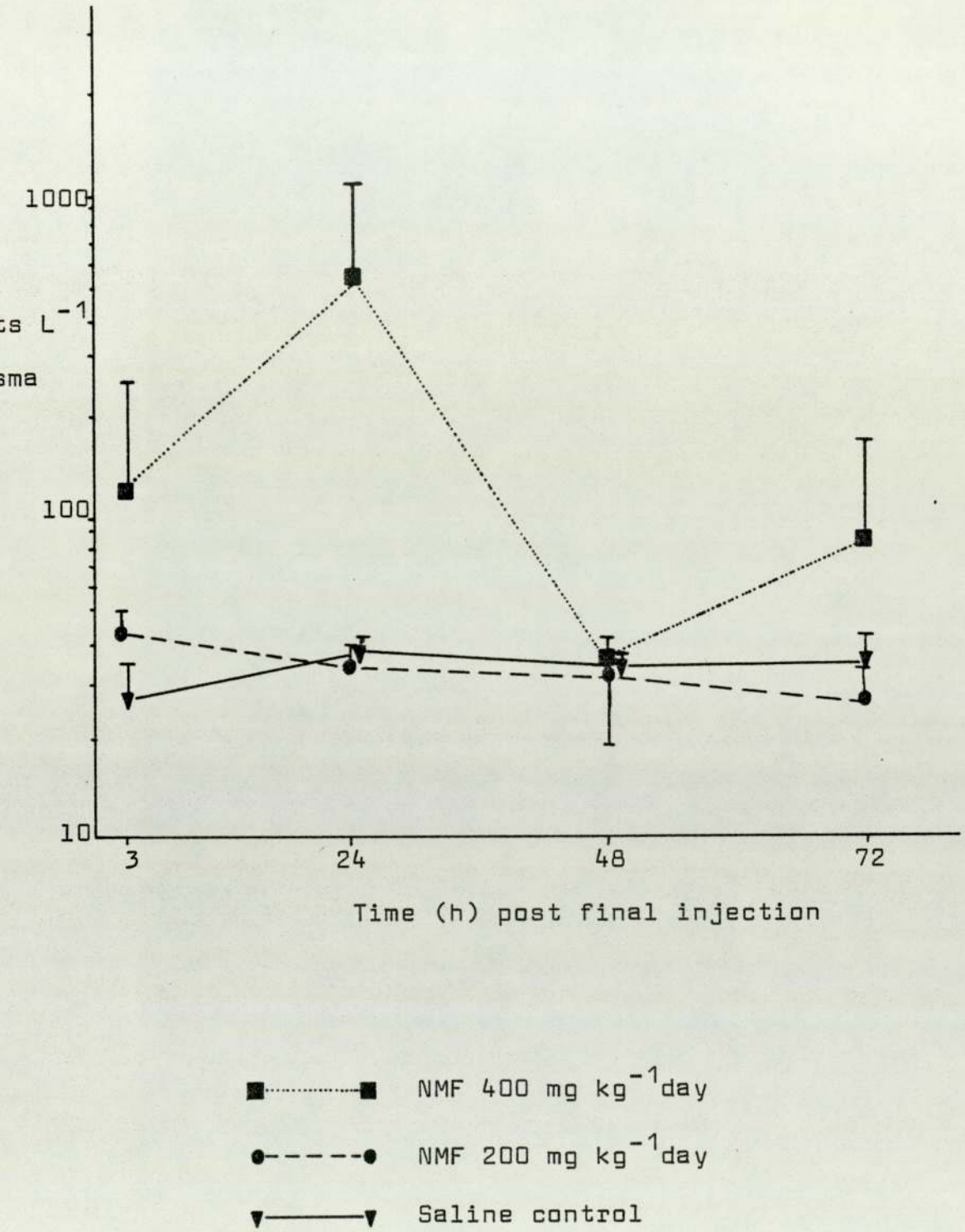


Fig 10.6 Plasma levels of alanine aminotransferase in female BDF$_1$ mice after a chronic (10 day) schedule of NMF.

From the schedule dependency study of NMF against the M5 sarcoma (table 9.1) it was concluded that chronic low dose schedules are therapeutically superior to high dose acute treatments. From table 10.3 it is also apparent that larger total doses of the drug may be administered without subsequent hepatotoxicity if a chronic schedule, as opposed to an acute treatment, is employed. These considerations contributed to

Schedule days of injection	LD_{10} mg $kg^{-1}day^{-1}$	Minimum hepatotoxic dose mg $kg^{-1}day^{-1}$	Total dose to render hepatotoxicity mg kg^{-1}
1	2200	800	800
1 - 10	485	400	4000

Table 10.3 Relationship of lethal dose levels to hepatotoxic levels in BDF_1 mice.

the rationale for testing this agent on a chronic alternate day schedule in the phase 1 clinical trial conducted in 1982 at the Charing Cross Hospital in London.

Since low dose chronic schedules appear to result in optimal antitumour activity yet reduced hepatotoxicity this may be an indication that the two effects result from separate events. Further evidence that this may be so is provided by the observation that in some studies DMF was found to be as hepatotoxic as NMF in both rats[191] and mice[195] yet is very markedly less active than NMF if active at all (chapter 9) against murine tumours.

Chapter 11 N-Methylformamide (NMF) in Combination Chemotherapy

11.1 Introduction

Two combinations containing NMF were studied. The first, that of cyclophosphamide and NMF, was designed to utilize the lack of bone marrow suppression of NMF. The second combination tested was that of HMM and NMF. Stevens has suggested that dissolution in NMF may be a useful formulation for poorly water-soluble antitumour agents.[130] HMM which is too water-insoluble to be administered by the i.v. route was thus studied in combination with NMF in which it is more soluble.[196] Since this was only a preliminary experiment to determine whether the combination might be effective, the two drugs were administered separately.

11.2 The combination of cyclophosphamide and NMF

As with other alkylating agents the major toxicity of cyclophosphamide is against the bone marrow and especially towards the white blood cell precursors.[197] Since NMF apparently does not possess this toxicity it seemed possible that 'therapeutic synergism' might be achieved in a combination of these two agents. Venditti[198] has defined therapeutic synergism as being 'the effective combining of agents to produce an improved therapy' and thus if a greater antitumour response could be obtained by the combination without any increased bone marrow toxicity then a synergistic effect would have been demonstrated.

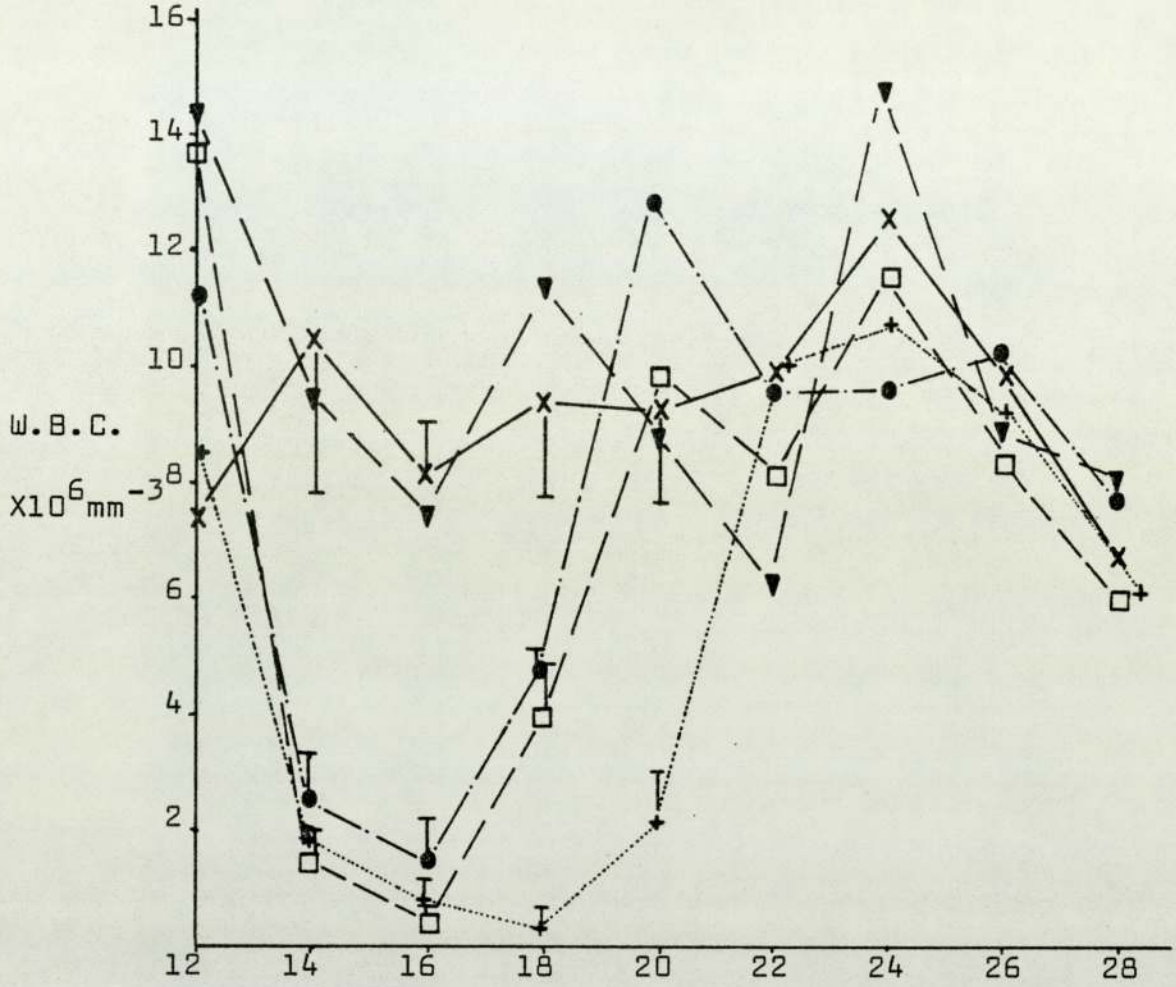
An experiment was designed in which mice with M5 tumours received a single injection of cyclophosphamide, 12 days after implantation of the tumour. Three days later when the marrow cells and colony forming units were known to be particularly sensitive to treatment,[199] a 10 day schedule of NMF injections was initiated. White blood cells, platelets and tumour volumes were monitored. Combinations of the two drugs were compared with the single agents given alone and also with a two injection schedule of cyclophosphamide, the second being administered 3 days after the first. Two doses of cyclophosphamide were selected, the LD_{10} and half the LD_{10} values (i.e. 320 mg kg^{-1} and 160 mg kg^{-1} respectively) and the dose of NMF chosen was 200 mg $kg^{-1}day^{-1}$ for 10 days which corresponds to approximately half the LD_{10} value. Thus the treatments compared were those shown in table 11.1.

White blood counts, tumour volume measurements and platelet counts are illustrated in figs. 11.1 and 11.2 and recorded in appendices 11.1 - 11.3.

In the white blood count determinations, NMF alone (schedule C) produced no change from control values whereas cyclophosphamide alone at either its LD_{10} value (schedule A) or half LD_{10} dose (schedule B) produced a precipitous fall. When NMF was combined with cyclophosphamide (schedules D and E) no reduction resulted over and above that induced by cyclophosphamide alone. Repeat treatment with cyclophosphamide

Schedule	Treatment	NMF dose (mg $kg^{-1}day^{-1}$)	Cyclophosphamide dose (mg $kg^{-1}day^{-1}$)
A	Cyclophosphamide day 12 only	-	320
B	Cyclophosphamide day 12 only	-	160
C	NMF days 15 - 24	200	-
D	Cyclophosphamide day 12/NMF days 15 - 24	200	320
E	Cyclophosphamide day 12/NMF days 15 - 24	200	160
F	Cyclophosphamide days 12 and 15	-	160
G	Untreated control	-	-

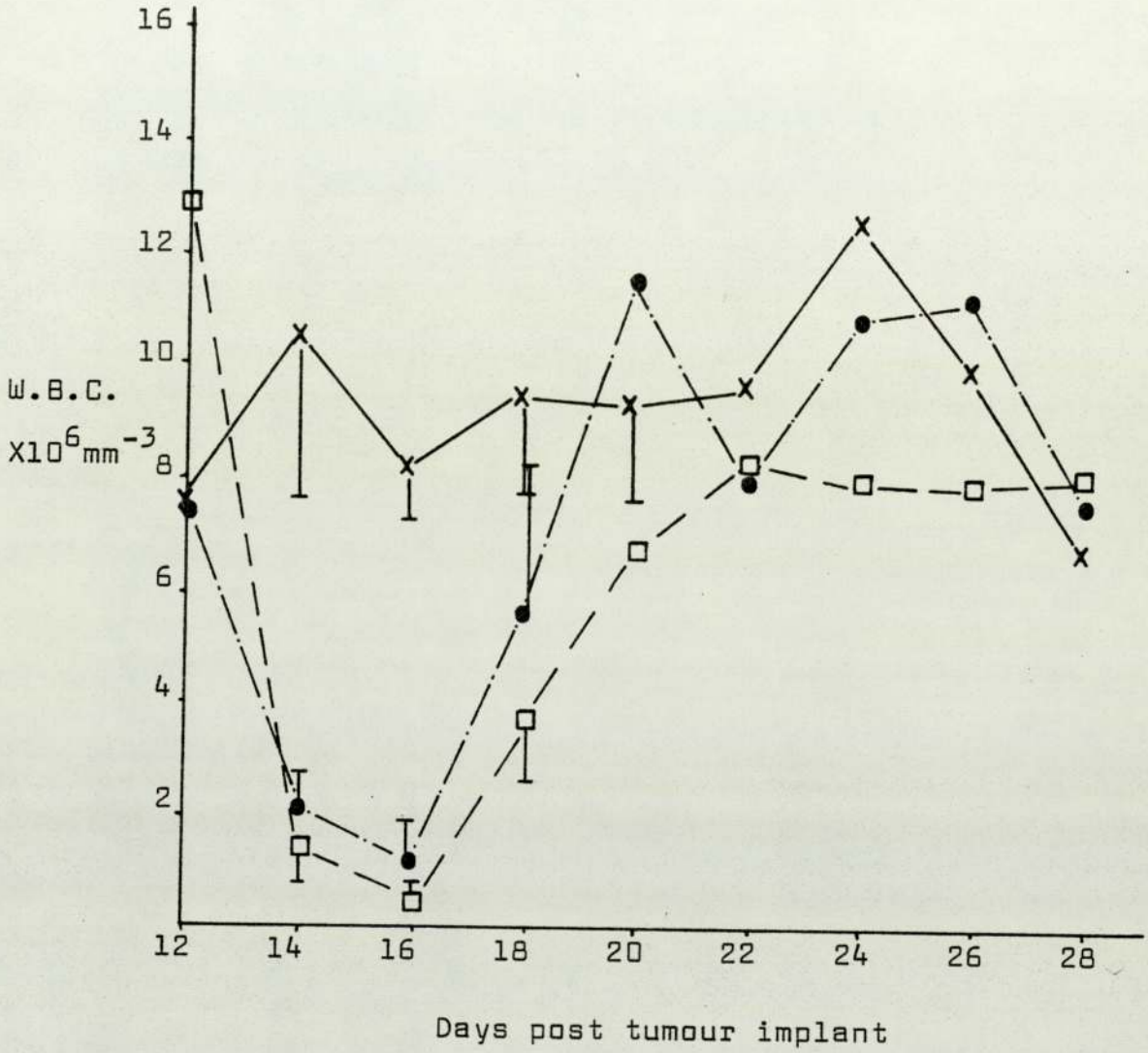
Table 11.1 Treatments used in the cyclophosphamide/NMF combination experiments



	Treatment	Dose*	Schedule
□— —-□	Cyclophosphamide	320	Day 12
●—·—●	Cyclophosphamide	160	Day 12
+·····+	Cyclophosphamide	160	Days 12 and 15
▼— —▼	NMF	200	Days 15 - 24
X——X	Control	-	

* = mg $kg^{-1}day^{-1}$

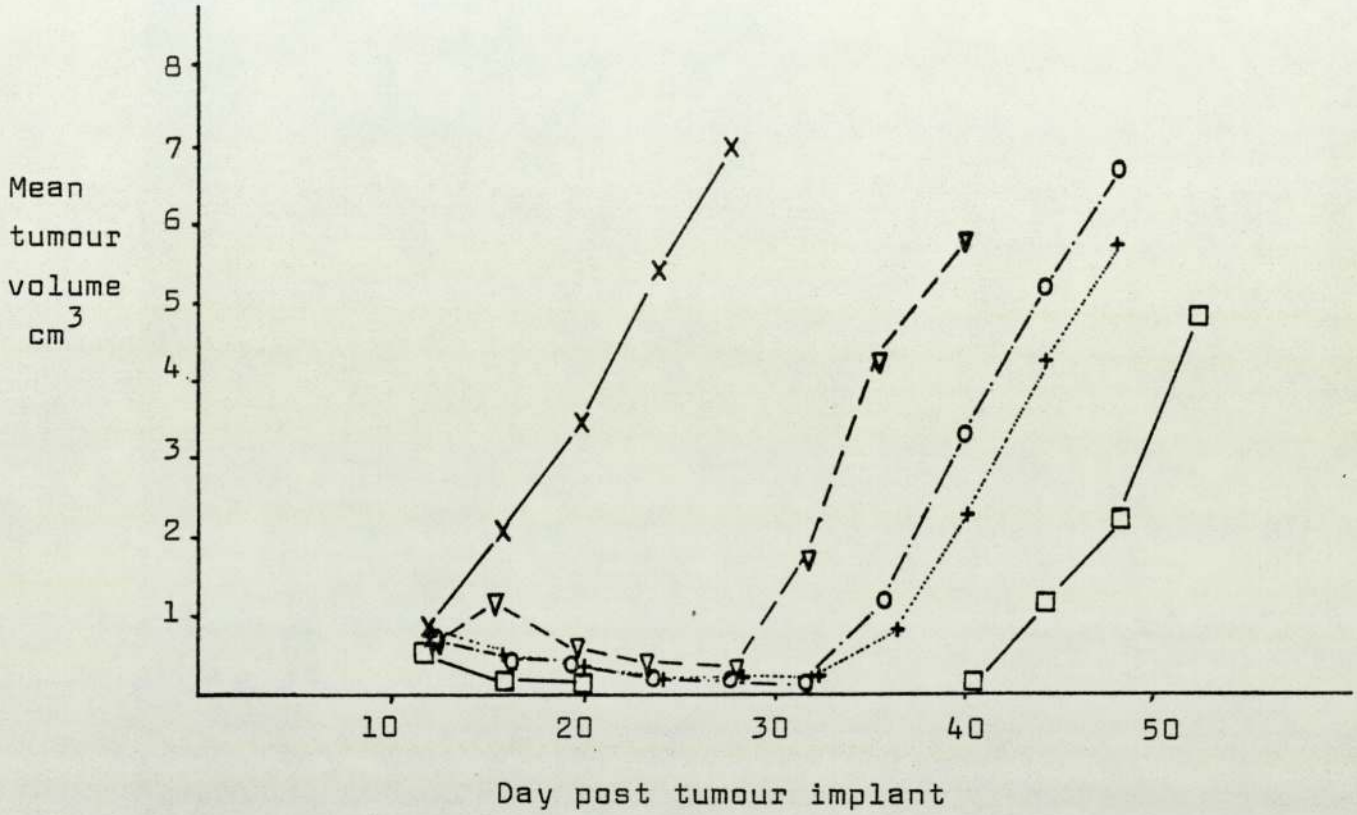
Fig 11.1.a Peripheral white blood cell counts (W.B.C ± s.d.) of mice treated with Cyclophosphamide/NMF.



	Treatment	Dose*	Schedule
□— — -□	Cyclophosphamide	320	Day 12
	NMF	200	Days 15 - 24
●—·—·—●	Cyclophosphamide	160	Day 12
	NMF	200	Days 15 - 24
X——X	Control	-	

* = mg $kg^{-1}day^{-1}$

Fig 11.1.b Peripheral white blood cell counts (W.B.C ± s.d.) of mice treated with Cyclophosphamide/NMF.



	Treatment	Dose*	Schedule
o—·—·o	Cyclophosphamide	320	Day 12
+·····+	Cyclophosphamide	160	Days 12 and 15
▽----▽	NMF	200	Days 15 - 24
□——□	(Cyclophosphamide	320	Day 12
	(NMF	200	Days 15 - 24
X——X	Control	-	

* = mg $kg^{-1}day^{-1}$

Fig 11.2 Mean tumour volumes of M5 sarcoma-bearing mice treated with the Cyclophosphamide/NMF combination

(schedule F) extended the leukopenia induced by the initial treatment. The depression induced by schedule F is also greater than that induced by either schedules A or B (fig. 11.1).

The delay time to reach the arbitrary mean tumour volume of 3.5 cm^3 was used to compare the tumour volume inhibitions of these schedules. These values are recorded in table 11.2. Consideration of the delay times indicate the most promising schedules to be the combination treatments of D and E. Both of these were superior to A, B or C. The repeat cyclophosphamide treatment (schedule F) produced a useful inhibition, but it was still inferior to either of the combinations.

Schedule	Time to reach 3.5 cm^3 (days)	% Increase in delay time
A	40.4	102
B	35.6	78
C	34.7	74
D	49.8	149
E	46.8	134
F	42.3	112
G	20.0	-

Table 11.2 Comparison of the tumour volume delay times for the different schedules employed in the Cyclophosphamide/NMF combination experiment

The platelet counts were not influenced by the administration of the drugs except for a slight rise induced by NMF at the end of its chronic schedule.

Several points may therefore be made. Firstly treatment with NMF initiated 3 days after treatment with high dose cyclophosphamide produced no increased leukopenia over and above that of the cyclophosphamide treatment alone. However an improved antitumour effect against the M5 sarcoma was observed. Secondly a repeat injection of cyclophosphamide 3 days after the first results in an extended leukopenia and yet does not produce as potent an antitumour effect as the addition of NMF to the initial injection of cyclophosphamide. Thirdly whereas treatment with cyclophosphamide at 320 mg kg^{-1} killed one mouse out of ten animals, treatment with the same dose of cyclophosphamide plus NMF at 200 mg kg^{-1} for 10 days did not result in any deaths suggesting that the mortality for the combination is no worse than for cyclophosphamide alone.

The plasma levels of the enzymes SDH, ALT and AST after treatment of the combination were also examined in order to determine whether the combination of drugs possessed hepatotoxicity over and above that of NMF alone. Results are tabulated in appendix 11.4 and no elevations relative to control values were observed. This indicates that the combination is not hepatotoxic at these dose levels. It is feasible that if the two drugs were used in combination in patients that the hepatotoxicity of NMF might interfere with

the metabolic activation of cyclophosphamide. Although a possibility it has not prevented potential hepatotoxins such as methotrexate[200] or 6-mercaptopurine[201,202] being used in combination with cyclophosphamide.

In conclusion, since an increased antitumour response was obtained without a concurrent increase in bone marrow toxicity or overall hepatotoxicity, the combination of cyclophosphamide and NMF may be said to be synergistic.

11.3 The combination of HMM and NMF

The combination of HMM and NMF was also investigated. HMM almost certainly requires metabolic activation (chapter 8) and this process occurs primarily in the liver. Therefore there is a possibility that the biochemical processes leading to the hepatotoxicity of NMF interfere with the metabolism of HMM. A preliminary experiment was conducted to investigate this factor.

Mice were first treated with NMF for five consecutive days at doses of 400 or 200 mg $kg^{-1}day^{-1}$. The livers of these mice were removed and their ability to demethylate HMM and aminopyrine, as measured by the production of formaldehyde, examined. The results are shown in table 11.3; values are given relative to the ability of saline treated livers to demethylate the substrates.

Substrate	Conc. (mM)	% Demethylation *		
		NMF pretreatment 400 mg $kg^{-1}day^{-1}$ X 5	NMF pretreatment 200 mg $kg^{-1}day^{-1}$ X 5	Saline control
HMM	0.5	55 ($\pm$ 7)	114 ($\pm$ 33)	100 ($\pm$ 26)
Aminopyrine	5.0	80 ($\pm$ 7)	104 ($\pm$ 16)	100 ($\pm$ 25)

* relative to saline controls

Table 11.3 The ability of the 10,000 x g supernatant fraction of the liver to demethylate various substrates after exposure to NMF

NMF pretreatment at a level of 400 mg $kg^{-1}day^{-1}$ for 5 days reduced the ability of the liver (10,000 x g supernatant fraction) to demethylate HMM, however at the dose of 200 mg $kg^{-1}day^{-1}$ over 5 days, no difference from control values was observed. Little effect upon aminopyrine metabolism was observed at either of these doses. Therefore in vivo NMF at high doses may well reduce the rate of HMM metabolism. It is not yet known whether this would be disadvantageous or not.

Whereas myelosuppression is clearly the major toxicity of cyclophosphamide, neurological and gastrointestinal toxicities as well as myelosuppression[76] all represent toxicities of HMM which may be dose limiting in a particular patient. Therefore in the combination experiments, in the absence of any single suitable parameter, mortality was chosen as the end point of toxicity and compared with the antitumour response obtained against the M5 sarcoma.

In table 11.4 (appendix 11.5) the results of a series of treatments of NMF alone, HMM alone and various combinations of the two drugs tested against the M5 sarcoma are summarized. Mean tumour volume inhibition as measured by the delay time to reach 3.5 cm^3 was used. The number of deaths attributable to the toxicity of the treatment is recorded. Comparison of treatment A which was toxic, with a very large weight loss, with say schedules J and K where no deaths occured shows that the two combinations (J and K) elicited a slightly better or

	NMF $mgkg^{-1}day^{-1}$	HMM $mgkg^{-1}day^{-1}$	No.dead / Total	Mean body weight change (g)	Day to reach 3.5 cm^3	Delay to * reach 3.5 cm^3	‡ % I.D.T.
A	400	-	1/5	-4.5	43.4	24.4	128
B	300	-	0/5	-0.9	39.8	20.8	109
C	200	-	0/5	+1.5	35.4	16.4	86
D	100	-	0/5	+2.2	29.6	10.6	56
E	50	-	0/5	+2.6	24.8	5.8	31
F	-	150	5/5	-	-	-	-
G	-	100	0/5	+0.5	36.6	17.6	93
H	-	50	0/5	+2.7	29.2	10.2	54
I	-	25	0/5	+2.9	22.6	3.6	19
J	300	50	0/5	+1.2	46.0	27.0	142
K	200	100	0/5	+0.4	44.4	25.4	134
L	200	50	0/5	+1.9	42.6	23.6	124
M	100	100	0/5	+0.8	39.1	20.1	106
N	100	50	0/5	+1.0	34.6	15.6	82
O	50	100	0/5	+1.0	35.5	16.5	87
P	50	50	0/5	+2.1	33.5	14.5	76
Control	-	-	0/9	+3.6	19.0	-	-

* Relative to control ‡ % IDT = % Increase in delay time

Table 11.4 Comparison of the tumour volume delay times for the different schedules employed in the HMM/NMF combination experiment.

equal antitumour response with less toxicity. Similarly treatment G is about the optimal non-lethal schedule for HMM alone whereas a combination such as K produces a superior response. Comparison of schedules O and G however demonstrate that the degree of synergism is very small and the effects are little more than additive; such a combination is unlikely to be clinically useful.

It is not possible to say whether the hepatotoxicity of NMF influenced the antitumour activity of HMM in this experiment. The responses of the combinations compared to the single agents was not very marked which might have resulted if there had been interference of the metabolism of HMM by NMF. Alternatively it is possible that the two agents possess a similar mode of action and thus the overall antitumour effect would only be expected to be additive.

CHAPTER 12 Comparison of the antitumour activity and metabolism of hexamethylmelamine (HMM) and N-methylformamide (NMF)

Although both HMM and NMF possess a requirement of a methylamino group for optimal antitumour activity (sections 7.3 and 9.3) it is not at all certain that they exert antineoplastic activity by a similar mechanism.

The two agents share both similarities and dissimilarities with respect to their antitumour activity. Both agents are very much more effective against human xenografts implanted in mice than they are against murine tumour models.[171-173,119] In both cases the ethyl analogues (HEM and NEF) are inactive against all murine models tested (sections 7.3 and 9.3, ref. 98) and both HMM and NMF exert optimal activity against murine tumours (such as the M5 sarcoma) when administered on a chronic as opposed to an acute schedule (sections 7.2 and 9.2). However while melamine, the homologue of HMM devoid of methyl groups, is inactive against the M5 sarcoma and Sarcoma 180 models (section 7.3 and ref. 9.8), formamide, the desmethyl analogue of NMF, retains a small degree of activity against both of these systems (section 9.2). Also DMF, the N-methyl analogue of NMF, might be expected to be as active, if not more so, than NMF if a mechanism analogous to that of HMM were operating. Brindley has postulated that differences in bioavailability may account for the inactivity of these analogues.[207] The AUC values in the plasma for HMM and NMF are greater than those for HEM and NEF and DMF respectively

after administration of equimolar doses of these compounds.[207] Thus the preference of the methyl over that of the ethyl moiety for antineoplastic activity in these agents may be at least partially based on pharmacokinetic reasons.

Important differences in metabolism are found between HMM and NMF.

In vitro HMM readily undergoes N-methyl hydroxylation upon incubation with liver microsomes and this process results in an increase in cytotoxicity towards tumour cells (section 8.2). NMF, however, does not undergo appreciable metabolism in the presence of microsomes.[126]

In vivo HMM is extensively demethylated and N^2,N^4-dimethylmelamine and monomethylmelamine emerge as the major urinary metabolites.[92,93] No unchanged HMM is recovered. NMF is also demethylated but to a lesser degree as manifested by the appearance of a small amount of formamide on G.C. analysis of the urine. However over 26% NMF is recovered unchanged in the urine.[207]

For HMM in vivo antitumour activity and in vitro cytotoxicity correlate with the demethylation process (sections 8.2 and 8.3, ref. 98). HMPMM, an intermediate metabolite in this process both in vitro and in vivo, is a very much more cytotoxic species than HMM in vitro and demonstrates antitumour activity in vivo (sections 8.2 and 8.3). HMF, the analogous

metabolite of NMF, is a very much less toxic species than its methyl precursor *in vivo* and demonstrates markedly reduced antitumour activity when administered *per se* *in vivo* (section 9.3). It is unclear how cytotoxic this species is *in vitro* because synthetic samples are contaminated with formaldehyde (section 9.3). There is at present no evidence to suggest that the metabolic demethylation is related to the antineoplastic activity of NMF. Indeed the process has been suggested to represent a detoxification pathway for this compound.[127] If it were important then it is necessary to explain why DMF possesses only a small degree of antitumour activity and yet is N-demethylated more readily *in vivo* than NMF.

The observation that formamide, which does not possess a methyl group, retains some antitumour activity has to be considered carefully. It is feasible that NMF may possess a different mode of action to that of HMM. The drug is capable of redifferentiating certain cell lines *in vitro* and such a process may be related to its antineoplastic activity.[186] However this hypothesis is difficult to defend in the light of the finding that agents such as DMSO and NNN′N′-tetramethylurea are also capable of inducing redifferentiation and yet are inactive against most murine tumour models.

It is interesting that NMF is able to elicit antitumour responses at doses which do not damage the bone marrow (section 10.4) This may indicate that it is unlike the antiproliferative agents in its mode of action.

In conclusion therefore the available evidence does not yet indicate that the two agents share a common mode of action.

SECTION 3

MATERIALS AND METHODS

Chapter 13 Experimental : Chemistry

13.1 Notes on instruments and materials

1. All melting points are reported uncorrected.
2. 'Ethanol' refers to 95% ethanol.
3. Infra red spectra were recorded on a Pye-Unicam SP200 spectrophotometer as KBr discs.
4. Ultra violet spectra were recorded on a Pye-Unicam SP8000 spectrophotometer.
5. ^{1}H n.m.r. spectra were recorded on a Varian EM 360 A (60 MH_3) spectrophotometer. All spectra were recorded in deuterated chloroform ($CDCl_3$) or water (D_2O). The signals were assigned in p.p.m. downfield of tetramethylsilane (δ).
6. The mass spectra were recorded on a Micromass 128 single focusing mass spectrometer.
7. Microanalyses were carried out at Elemental Micro-Analyses Limited and at the Department of Chemistry, Aston University.

13.2 Synthesis of 2,4,6-trisubstituted-s-triazines

The following compounds were synthesized using published methods. All structures were confirmed by i.r., n.m.r. and mass spectroscopy.

2-Chloro-4,6-bis(dimethylamino)-s-triazine (64), m.p. 66°-68° (lit.[148], m.p. 66°-68°).

2-Azido-4,6-bis(dimethylamino)-s-triazine (65), m.p. 105°-107° (lit.[149], m.p. 104°-106°).

2-Amino-4,6-bis(dimethylamino)-s-triazine (62), m.p. 224°-226° (lit.[149], 220°-222°).

2,4,6-Tris(dimethylamino)-s-triazine (12), m.p. 171°-173° (lit.[146], 170.5°-173°).

2,4-Bis(dimethylamino)-6-methylamino-s-triazine (33), m.p. 100°-104° (lit.[146], 98°-103°).

2,4-Bis(dimethylamino)-6-(N-hydroxy-N-methylamino)-s-triazine (66), m.p. 109°-111° (lit.[145], 110°-111°).

2,4-Bis(dimethylamino)-6-hydrazino-s-triazine (67), m.p. 146°-148° (lit.[149], m.p. 148.5-152.5°).

2-Chloro-4,6-bis (methylamino)-s-triazine (68), m.p. >335° (lit.[148], >335°).

2-Dimethylamino-4,6-bis(methylamino)-s-triazine (69), m.p. 91°-92° (lit.[146], 91°-92°).

2,4,6-Tris(methylamino)-s-triazine (70), m.p. 129°-131° (lit.[146], 129°-131°).

N-[4,6-Bis(dimethylamino)-s-triazin-2-yl]-N-methylformamide (71), m.p. 128°-131° (lit.[150],128°-131°).

{[4,6-Bis(dimethylamino)-s-triazin-2-yl]-methylamino}-methanol (37), m.p. 120°-123° (lit.[148],119°-123°).

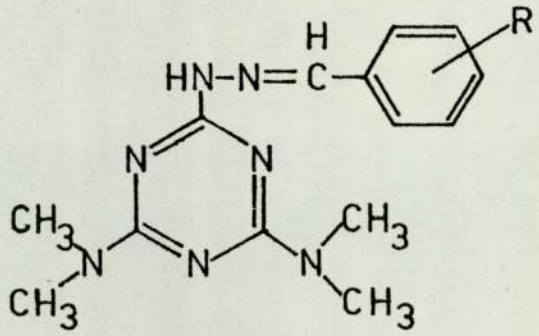
$N^2,N^{2'}$-Methylenebis(N^2,N^4,N^4,N^6,N^6-pentamethylmelamine) (72), m.p. 195°-196° (lit.[146], 195°-196°).

13.3 Synthesis of the novel 2-(substituted benzylidenehydrazino)-4,6-bis(dimethylamino)-s-triazines (Table 13.1)

2,4-Bis(dimethylamino)-6-hydrazino-s-triazine (39g; 0.2 mol) was dissolved in ethanol (200 ml). The appropriately substituted benzaldehyde (0.2 mol) was added and the solution heated under reflux for 1h. This was allowed to cool to room temperature whereupon the 2-substituted benzylidenehydrazino-4,6-bis(dimethylamino)-s-triazine crystallized from the solution. This was then further recrystallized from ethanol. Reaction was essentially complete with yields in the range 95 - 100%.

13.4 Synthesis of the novel 3-aryl-5,7-bis(dimethylamino) -s-triazolo [4,3-a] -1,3,5-triazines (Table 13.2)

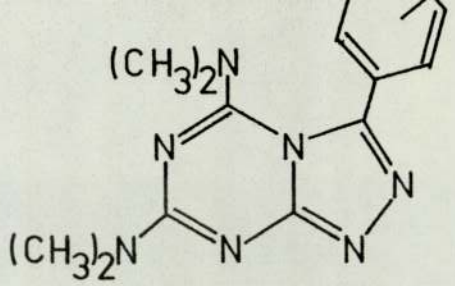
The 2-substituted benzylidenehydrazino-4,6-bis(dimethylamino) -s-triazine (2.0 g) was dissolved in either chloroform or dichloromethane (50 ml). Lead tetraacetate (4.0 g) was added portionwise to this solution and the reaction mixture stirred at room temperature for 2h. Water (50 ml) was added and stirring continued for a further $\frac{1}{2}$h. The aqueous layer was neutralized by the addition of sodium bicarbonate and the chloroform layer separated off. This chloroform layer was evaporated to dryness under reduced pressure to yield the s-triazolo [4,3-a] -1,3,5-triazine. These were recrystallized from a chloroform/methanol solution. The yields were 40 - 55%.



(76)

R	Compound Number	Appearance	M.p.	Elemental Analysis %					
				Expected			Found		
				C	H	N	C	H	N
-H	76a	White crystals	150°-153°						
o-NO_2	76b	Yellow crystals	156°-158°	50.9	5.5	33.9	50.7	5.6	33.8
p-NO_2	76c	Yellow crystals	224°-227°	50.9	5.5	33.9	50.7	5.5	33.7
o-Cl	76d	White crystals	138°-140°	52.6	5.6	30.7	52.2	5.7	30.4
m-Cl	76e	White crystals	158°-160°	52.6	5.6	30.7	52.8	5.7	30.9
p-Cl	76f	White crystals	207°-209°	52.6	5.6	30.7	52.5	5.8	30.7

Table 13.1 The 2-substituted benzylidenehydrazino-4,6-bis(dimethylamino)-s-triazines



(77)

R	Compound Number	Appearance	M.p.	Elemental Analysis %					
				Expected			Found		
				C	H	N	C	H	N
-H	77a	White crystals	207^{o}-209^{o} lit.[153] 172^{o}-173^{o}	-	-	-	-	-	-
o-NO_2	77b	Orange crystals	250^{o}-252^{o}	51.2	4.9	34.1	51.6	5.0	34.2
o-Cl	77c	White crystals	222^{o}-224^{o}	52.9	5.0	30.9	52.0	5.0	30.1
m-Cl	77d	White crystals		52.9	5.0	30.9	52.6	5.0	30.3
p-Cl	77e	White crystals	197^{o}-199^{o}	52.9	5.0	30.9	52.2	5.7	30.1

Table 13.2 The 3-aryl-5,7-bis(dimethylamino)-s-triazolo [4,3-a]-1,3,5-triazines

13.5 Synthesis of novel 2-aryl-5,7-bis(dimethylamino)-s-triazolo[2,3-a]-1,3,5-triazines (Table 13.3)

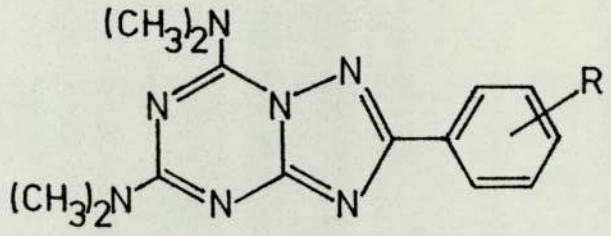
The 3-aryl-5,7-bis(dimethylamino)-s-triazolo [4,3-a]-1,3,5-triazine was dissolved in a 5% methanolic solution (100 ml) of sodium hydroxide. The reaction was stirred at room temperature for 2h whereupon the 2-aryl-5,7-bis(dimethylamino)-s-triazolo [2,3-a]-1,3,5-triazine precipitated out of solution. This was subsequently recrystallized from chloroform/methanol. The conversion in all cases was found to be between 80 and 90%.

13.6 Synthesis of 2-(o-aminophenyl)-5,7-bis(dimethylamino)-s-triazolo [2,3-a]-1,3,5-triazine (78f) (Table 13.3)

The nitro analogue (78b) (2.0 g) was dissolved in ethanol (200 ml). The solution was then hydrogenated at room temperature over 3 days using a 5% palladium/carbon catalyst (0.3 g). The product is insoluble in ethanol and dissolution in chloroform followed by filtration removed the catalyst. The solvent was removed under reduced pressure to afford the amine (78f) in 95% yield. This was recrystallized from chloroform/methanol.

13.7 Synthesis of 2-(o-azidophenyl)-5,7-bis(dimethylamino)-s-triazolo[2,3-a]-1,3,5-triazine (78g) (Table 13.3)

A solution of the amine (78f) (2.0 g) in water (10 ml) was cooled to $0^{o}C$ and acidified with 10N-hydrochloric acid (2 ml). Vigorous stirring and the low temperature were maintained throughout the following reaction steps. An aqueous solution of sodium nitrite (0.7 g in 5 ml) was added in small portions



(78)

R	Compound Number	Appearance	M.p.	Elemental Analysis %					
				Expected			Found		
				C	H	N	C	H	N
-H	78a	White crystals	232^{o}-234^{o} lit.[153] 230^{o}-231^{o}	-	-	-	-	-	-
o-NO_2	78b	Cream-yellow crystals	246^{o}-249^{o}	51.2	4.9	34.1	51.4	5.0	34.7
o-Cl	78c	White crystals	205^{o}-208^{o}	52.9	5.0	30.9	52.8	5.1	30.8
m-Cl	78d	White crystals	211^{o}-213^{o}	52.9	5.0	30.9	52.7	5.0	30.8
p-Cl	78e	White crystals	170^{o}-172^{o}	52.9	5.0	30.9	52.0	5.0	30.4
o-NH_2	78f	Cream crystals	219^{o}-221^{o}	56.4	6.0	37.3	56.2	6.0	37.3
o-N_3	78g	Brown crystals	178^{o}-180^{o}	51.9	4.9	43.2	51.6	5.0	43.0

Table 13.3 The 2-aryl-5,7-bis(dimethylamino)-s-triazolo[2,3-a]-1,3,5-triazines

over 0.5h. The mixture was stirred for a further 1h at 0°C. Sodium azide (1.0 g) was then added and the solution stirred for a further 1h and allowed to warm up to room temperature. Chloroform was added in order to extract the azide (78g) after neutralization of the aqueous layer with sodium bicarbonate. The chloroform was removed under reduced pressure and the azide (95% yield) recrystallized from chloroform/methanol.

Chapter 14 Biological Materials and Methods

14.1 Drugs used in the screening studies

14.1 (a) Purchased

The following compounds were purchased from the sources indicated.

Sigma Chemical Company, Poole

Cyclophosphamide

5-Fluorouracil

Aldrich Chemical Company, Gillingham

Formamide

N-Methylformamide

NN-Dimethylformamide

N-Methylacetamide

NN-Dimethylacetamide

NN′-Dimethylurea

NN′-Dimethylthiourea

NNN′N′-Tetramethylurea

NNN′N′-Tetramethylthiourea

Other sources

Chlorambucil - Burroughs Wellcome & Co., London

Cis-Diamminedichloroplatinum (II) - Johnson Matthey Research, Reading.

CCNU - N.C.I.

Methotrexate - Lederle Laboratories Division, Cyanamid Ltd., Gosport.

DTIC - Dome Laboratories, Slough

Treosulphan - Leo Laboratories Ltd., Bucks

Adriamycin - Farmitalia, Milan

N,N,N′,N′-Tetramethylguanidine - Koch-Light Laboratories Limited, Colsbrook.

Procarbazine - Roche Products Ltd., Welwyn Garden City.

14.1.(b) Gifts

The following compounds were supplied as gifts from various members of the C.R.C. Experimental Chemotherapy Laboratories, Pharmacy Dept., Aston University, Birmingham.

3-O-[4,6-Bis(dimethylamino)-s-triazin-2-yl]-D-glucopyranose, 2-amino-N-[4,6-bis(dimethylamino)-s-triazin-2-yl]-2-deoxy-D-glucopyranose, 2-O-[4,6-bis(dimethylamino)-s-triazin-2-yl]-β-glucopyranoside, and N-ethylformamide were gifts from Dr. R.J.Simmonds. N-Hydroxymethylformamide was kindly provided by Dr. A. Gescher and N-hydroxymethyl-N-methylformamide by Mr. E.N.Gate. N-Methyltrifluoroacetamide was supplied by Dr. M.D.Threadgill.

14.1.(c) Synthesized

All other drugs were synthesized as described in chapter 13.

14.2 Miscellaneous compounds

The following compounds were purchased from the sources indicated.

B.D.H.Chemicals Ltd., Atherstone

Acetylacetone

Dimethylsulphoxide

Glacial acetic acid.

Aldrich Chemical Company, Gillingham

3-Methyl-2-benzothiazolone hydrazone hydrochloride

Sigma Chemical Company, Poole

L-Alanine

L-Aspartate

Bovine serum albumin

Glucose-6-phosphate

Glucose-6-phosphate dehydrogenase

Heparin

α-Ketoglutaric acid

Lactate dehydrogenase

Malate dehydrogenase

Nicotinamide adenine dinucleotide phosphate

Nicotinamide adenine dinucleotide - reduced form

Pyridoxal-5′-phosphate

Fisons Scientific Apparatus, Loughborough

Ammonium acetate

14.3 Animals

Balb/c and BDF_1 mice were obtained from Bantin and Kingman Limited, Hull.

DBA/2, CDF_1 and BDF_1 mice were obtained from Charles Rivers Breeding Laboratories.

All animals were maintained in an animal house for at least one week to acclimatize. They were fed on water and Heygates modified 418 breeding diet ad libitum.

14.4 Media

RPMI-1640 medium (with 25mM Hepes and L-glutamine) and also horse serum were obtained from Gibco Europe.

14.5 Tumour cell and blood cell count apparatus

White and red blood cell pipettes were obtained from Weber Scientific International as was also the BS.748 Neubauer haemocytometer. A CK Olympus Tokyo microscope and ZB-1 Coulter Counter were employed.

14.6 Earl's buffer solution

Sodium chloride	-6.80g
Sodium bicarbonate	-2.10g
Glucose	-2.00g
Potassium chloride	-0.40g
Magnesium sulphate heptahydrate	-0.14g
Calcium chloride	-0.28g
Sodium dihydrogen phosphate dihydrate	-0.14g
Distilled water	-to 1L

This buffer was adjusted to pH 7.4 by the addition of hydrochloric acid.

14.7 Tumour techniques

In tables 14.1 and 14.2 the parameters of the tumour systems employed are summarized.

14.7(a) Solid tumour maintenance (table 14.1)

The tumour was excised from a donor mouse under aseptic conditions and placed into saline. The tumour was then either homogenized (in a Camlab 563 C homogenizer fitted with a teflon pestle), diluted to the appropriate concentration and reinjected into fresh mice or it was implanted by trochar as

TUMOUR	TYPE	MOUSE STRAIN	SOURCE OF TUMOUR	INOCULUM			Interval between passages (days)
				SITE	FORM	LEVEL	
M5076 (M5) RETICULUM CELL SARCOMA	Solid	BDF_1 (female)	Mario Negri Institute, Milan and Institute of Cancer Research, Sutton	s.c.flank or i.m.leg	Fragment or homogenate	$\sim 1mm^3$ or 10^6 cells	14 - 21
	Ascitic	BDF_1 (female)	Aston University	i.p.	Suspension	10^6	14 - 21
ADJ/PC6A (PC6) PLASMACYTOMA	Solid	Balb/c	Institute of Cancer Research, Sutton	s.c. flank i.m. leg	Fragment or homogenate	$\sim 1mm^3$ or 10^6 cells	14 - 21
P388 LYMPHOCYTIC LEUKAEMIA	Ascitic	DBA/2	Jules Bordet Institute, Brussels	i.p.	Suspension	10^6	7
L1210 LYMPHOCYTIC LEUKAEMIA	Ascitic	DBA/2	Jules Bordet Institute, Brussels	i.p.	Suspension	10^5	6 - 7
SARCOMA 180	Ascitic	BDF_1	Jules Bordet Institute, Brussels	i.p.	Pure ascites	Pure ascites (0.5 ml)	6 - 7

Table 14.1 Tumour maintenance

TUMOUR	TYPE	MOUSE STRAIN	NO. MICE /DOSE	INOCULUM			EXPERI-MENT TERMI-NATION DAY	EVALUATION PARAMETER
				SITE	FORM	LEVEL		
M5076 (M5) RETICULUM CELL SARCOMA	Solid	BDF_1 (female)	5 or 10	i.m. leg	Homogenate	10^6	24	Mean tumour volume inhibition
	Ascitic	BDF_1 (female)	5	i.p.	Suspension	10^6	90	Mean survival time
ADJ/PC6A (PC6) PLASMACYTOMA	Solid	Balb/c	5	i.m.leg	Homogenate	10^6	24	Mean tumour volume inhibition
P388 LYMPHOCYTIC LEUKAEMIA	Ascitic	CDF_1	5 or 6	i.p.	Suspension	10^6	60	Median survival time
L1210 LYMPHOCYTIC LEUKAEMIA	Ascitic	CDF_1	5 or 6	i.p.	Suspension	10^5	60	Mean survival time
SARCOMA 180	Solid	BDF_1	5 or 10	i.m.leg	Suspension	10^6	60	Mean tumour volume inhibition
	Ascitic	BDF_1	5 or 10	i.p.	Suspension	10^6	60	Mean survival time

Table 14.2 Tumour screening protocols

a fragment. Tumour lines were passaged for no more than 10 generations after which time they were destroyed and fresh tumour was obtained from a store maintained in liquid nitrogen.

14.7.(b) Ascitic tumour maintenance (table 14.1)

Ascitic cells were aspirated from the peritoneal cavities of donor mice. Cells were counted, diluted to the required concentration and injected i.p. into new mice. In the same manner as for the solid tumours, cell lines were destroyed periodically and fresh tumour obtained from a bank.

14.7.(c) Solid tumour screening assays (table 14.2)

Tumour fragments were excised from several tumour-bearing hosts under aseptic conditions. The fragments were pooled, placed into sterile saline and cut by scalpel into small pieces. These fragments were homogenized as above (14.7.a) at the slowest possible speed to minimize cell death. A sample of the resultant suspension was placed onto a haemocytometer grid and the cells counted under the microscope. This suspension was then diluted to the appropriate concentration and injected (0.1 ml: i.m.) into the left hind legs of recipient mice.

For the M5 sarcoma and Sarcoma 180 models drug injections were initiated 24h later according to the appropriate testing schedule. For the PC6 plasmacytoma drug injections were delayed until 14 days after tumour implantation at which time the tumour volumes measured 1.0 - 2.5 cm^3 in size.

Tumour growth was followed by measurement of the tumour volumes. The longest tumour diameter (length; mm) and the diameter perpendicular to this (width; mm) were first measured by vernier calipers. The approximate tumour volume was then calculated by the formula:

$$\text{Volume } (mm^3) = \frac{\text{length} \times (\text{width})^2}{2}$$

The mean tumour volume for a group of mice was then assessed.

Comparison of the tumour volumes of treated mice with those of untreated controls may be represented in several ways:

i) $\frac{T}{C} \times 100\%$

where T = Mean tumour volume of treated mice

C = Mean tumour volume of control mice

Thus 0% refers to complete absence of tumour in the treated mice and 100% refers to no difference between the volumes of treated and control mice.

ii) % Inhibition. This value is given by $100 - (T/C \times 100)$ % where T and C are defined as in i).

iii) % Increase in delay time. This is defined by

$$\frac{T - C}{C} \times 100\%$$

where T = Time (days) from implantation for tumour to reach a mean arbitrary volume for the treated mice.

C = Time (days) from implantation for tumour to reach a mean arbitrary volume for the control mice.

The ID_{90} is defined as that dose of the drug which will produce 90% inhibition of the mean tumour volume of treated mice as compared to the mean volume of control mice. LD_{10} and LD_{50} values were determined graphically using a range of doses varying from non-lethal to 100% mortality in tumour-bearing mice. The therapeutic index used for the M5 sarcoma and PC6 plasmacytoma experiments was defined as

$$\text{Therapeutic index (TI)} = \frac{LD_{50}}{ID_{90}}$$

The N.C.I. routinely adopt a value of 58% inhibition on the appropriate evaluation day as their criterion for activity of a drug against solid murine tumours. This value was also adopted in these studies.

The optimal dose was taken as that value tested nearest to the LD_{10} dose.

14.7.(d) Ascitic tumour screening assays (table 14.2)

Ascitic tumour cells were first extracted from the peritoneal cavities of tumour-bearing mice. These were then counted either by using a haemocytometer grid or with a Z81 Coulter Counter and diluted in sterile saline to the required concentration. Cells were stored on ice throughout all procedures. The tumour suspension (0.1 ml/mouse) was then injected i.p. into fresh mice. For both the P388 and L1210 leukaemia models drug injections were initiated 24h post implantation according to the appropriate dosing schedule. The parameter of evaluation of antitumour activity was increase in survival time of treated mice over that of controls.

Percentage increase in survival time (% IST)

$$= \frac{T - C}{C} \times 100\%$$

where T = Mean (L1210) or median (P388) death day of treated mice

C = Mean (L1210) or median (P388) death day of control mice.

The optimal dose is defined as that dose which produces the optimal increase in survival time. It should however not exceed the LD_{10} value.

14.8 Drug administration

14.8.(a) Solvents

The following solvents were used for the dissolution of drugs:

Saline

Saline/tween-80 (19/1)

Arachis oil

Arachis oil/dimethylsulphoxide (9/1)

Arachis oil/acetone (9/1)

14.8.(b) Route

Drugs were administered i.p. unless specifically stated otherwise.

14.8.(c) Schedules

Three types of drug scheduling were employed: acute, chronic or intermittent. The duration of the chronic dosing in the screening assays was arbitrarily taken as approximately half

the life span of the tumoured control mice, e.g. 17 days for the M5 sarcoma and 9 days for the Sarcoma 180.

In section 7.2 a chronic schedule of 8 injections day^{-1} (i.e. every 3h) on days 1,5,9 was employed in an attempt to maintain peak levels of HMM in M5 tumour-bearing mice on these days.

In section 8.4 formaldehyde was administered on days 1 - 5 then days 13 - 17. The drug was not administered between days 5 - 13 in order to allow local inflammation (caused by administration of the drug) to recover. HMM was injected by the same schedule since a comparison between the two drugs was being made.

14.9 PC6 plasmacytoma *in vitro* - *in vivo* bioassay (section 8.2)

Estimates of the *in vitro* cytotoxicity of the analogues of HMM were obtained by the following method.[203] PC6 ascites cells (2×10^6 ml^{-1}) were incubated in RPMI-1640 medium/horse serum (6/4) in the presence of the drug (5mM), liver microsomes (≡ 0.1g wet liver weight ml^{-1}) and either with or without the following cofactors; $MgCl_2$ (5mM), G6P (4mM), G6P dehydrogenase (15 units ml^{-1}) NADP (400 μM). These cofactors were present in order to generate NADPH (0.5mM). Microsomes were prepared from the livers of male Balb/c mice as described in 14.10. Incubations (4.0 ml) were performed for 2h at 37^o in open beakers in a shaking water bath. Subsequently 10^6 cells (0.5 ml suspension) were injected i.p.

into groups of 4 female Balb/c mice. After 7 days the animals were killed by cervical dislocation and the ascites removed by lavage of the peritoneum with saline. Inspection of the peritoneal cavity revealed no solid tumour deposits. Cells were counted using a Z81 Coulter Counter and the approximate cell kill determined by reference to the number of cells harvested from animals which had received serial dilutions of cells counted in identical fashion. In this assay a direct relationship between the number of cells injected and the numbers harvested was observed.

In the absence of cofactors activation is assumed not to occur.[98]

14.10 Preparation of liver microsomes

Microsomes were prepared from the livers of 3-week old mice according to the method of Schenkman and Cinti.[204] Liver homogenate (20% w/v) in 0.25 M sucrose was first centrifuged at 2-3,000 g for 5 min followed by centrifugation at 9,000 for 20 min at $4^{o}C$ to remove nuclei plus cell debris and mitochondria. The resultant supernatant was then centrifuged at 27,000 - 30,000 g for 20 min after addition of $CaCl_2$ (8mM) in order to obtain a microsomal pellet which was resuspended in RPMI-1640 medium/horse serum (6/4).

14.11 M5 sarcoma *in vitro* - *in vivo* bioassay (section 8.3)

This bioassay was conducted in identical fashion to the PC6 plasmacytoma bioassay (14.9) with the following changes. 2×10^7 cells ml^{-1} were employed in the incubations and

10^7 cells (0.5 ml incubate) were injected into each of 4 female BDF_1 mice. Microsomes were prepared from the livers of female BDF_1 mice. Cytotoxicity was evaluated in the following manner. The mean day of death of the mice receiving treated cells was compared with the days of death of mice receiving serial dilutions of M5 sarcoma cells, enabling an estimate of cell kill to be evaluated.

HMM (5mM) was incubated with microsomes at two different microsomal concentrations (200 and 400 mg ml^{-1}) in the present or absence of cofactors (14.9). HMPMM was incubated at both 2.5 mM or 5 mM with a microsomal concentration of 200 mg ml^{-1} in the absence of cofactors.

14.12 Lethal dose levels of NMF

NMF in sterile saline was administered to groups of 10 mice (Balb/c or BDF_1) at dose levels ranging from non-lethal to 100% mortality. At least 5 dose levels within this range were employed. A graph of log dose versus mortality was plotted and the line of best fit drawn. From this the LD_{10} and LD_{50} values were estimated. The drug was administered by the following routes: i.v. injection into a tail vein, i.p. administration, esophageal intubation (p.o.) or i.m. injection into the left hind leg.

14.13 Measurement of peripheral blood cell counts

While mice were under halothane anesthaesia, blood samples were collected from the tip of the tail into blood cell pipettes at various time intervals post drug injection.

Measurements of cell numbers were made in the following ways:

i) Erythrocytes: These were diluted in saline and counted on a Z81 Coulter Counter.

ii) White blood cells: These were diluted in a 1% acetic acid/saline solution stained with gentian violet and counted using a Weber B.S.A. improved Neubauer haemocytometer.

iii) Platelets: These were diluted in a sodium citrate solution (3g/100ml) stained with methylene blue and counted using the above haemocytometer.

Female Balb/c mice were employed for the studies of the peripheral blood counts after treatment with NMF (section 10.4). Female BDF_1 mice were used in studies of the combination of cyclophosphamide and NMF (section 11.2) since this mouse strain was the host of the M5 sarcoma.

14.14 Measurement of enzyme levels in the plasma

14.14.(a) Preparation of plasma samples

The animals were anaesthetized using a mixture of halothane, nitrous oxide and oxygen. Blood samples (1 ml) were collected by exsanguination from the abdominal aorta at the iliac bifurcation using a disposable syringe containing heparin (2,500 U ml^{-1}; 0.05 ml). Plasma was obtained by centrifugation of the blood samples for 1 min in an Eppendorf 5412 centrifuge (speed 7).

14.14.(b) Sorbitol dehydrogenase (SDH)

This was assayed according to a method described by Rose and Henderson.[205]

700 µl NADH (disodium salt, 355 µM) in TrisHCl (100 mM pH 6.6, 37°C) was placed in a reduced volume 1-cm pathlength cuvette. Plasma (100 µl) was added to this and the endogenous reactions allowed to proceed to completion (monitored by ΔA_{340}). 200 µl β-D-fructose (2.5M in 100 mM TrisHCl, pH 6.6, 37°) was added to initiate the SDH reaction and the absorbance at 340 nm was monitored. The activity of SDH in this system is given by

$$\text{SDH activity } (UL^{-1}) = 1608.\ \Delta A_{340}\ min^{-1}$$

where one unit (U) of SDH activity is equal to the reduction of 1µmol of fructose per litre per minute)

14.14.(c) Aspartate aminotransferase (AST)

This was assayed according to a method described by Kacmar and Moss.[206]

The following components were added to a reduced volume 1-cm pathlength cuvette; L-aspartate (767 µl; 228 mM), NADH (33 µl; 6.5 mM), pyridoxal-5'-phosphate (33 µl; 4.5 mM), malate dehydrogenase (16 µl; 36,000 U L^{-1}) lactate dehydrogenase (16 µl: 72,000 UL^{-1}) and plasma (67 µl). The cuvette and contents were then preincubated in the thermostatted cuvette compartment of the spectrophotometer at 37°C for 5 min to permit the endogenous side reactions to proceed to completion. The AST reaction was then initiated by adding α-ketoglutaric acid solution (67 µl; 225 mM) to the cuvette and measuring

the absorbance change at 340 nm for 10 min. The activity of AST in this system is given by

$$\text{AST activity } (UL^{-1}) = 2410.\ \Delta A_{340}\ min^{-1}$$

where one unit (U) of AST activity is equal to the oxidation of 1 μmol of NADH per litre per minute.

14.14.(d) Alanine aminotransferase (ALT)

The assay method for this enzyme is identical to that for AST except that L-alanine replaces L-aspartate as the amino group donor and lactate dehydrogenase replaces malate dehydrogenase as the indicator enzyme. Thus the mixture is as above (14.14.c) with L-alanine (767μl; 525 mM) being added, lactate dehydrogenase being present at a higher concentration (33 μl; 72,000 UL^{-1}) and L-aspartate and malate dehydrogenase not present

$$\text{ALT activity } (UL^{-1}) = 2410.\ \Delta A_{340}\ min^{-1}.$$

where one unit (U) of ALT activity is equal to the oxidation of 1 μmol of NADH per litre per minute.

14.15 Measurement of formaldehyde precursors generated during the metabolism of analogues of HMM in vivo

The colorimetric method employed to distinguish between formaldehyde and its precursors was the method as described by Ross[122] which is a modification of the method of Sawicki.[178]

Drugs were injected i.p. into male Balb/c mice at a dose of 0.48 mmol kg^{-1}. Control animals received vehicle only. Plasma was obtained as described in 14.14.a. Solutions (0.25 ml) containing either formaldehyde or HMPMM were added

to a 0.4% w/v 3-methyl-2-benzothiazolone hydrazone hydrochloride (MBTH) solution (0.25 ml) in quadruplicate at each concentration. Duplicate samples were then left at either $0^{o}C$ or $60^{o}C$ for 30 min when a 0.12% w/v solution of anhydrous iron III chloride (1.25 ml) was added. The mixtures were left for a further 5 min at room temperature and the reaction terminated by the addition of acetone (3.2 ml). The optical density of the resultant tetraazopentamethinecyamine complex was measured at 670 nm using a U.V. spectrophotometer.

Calibration curves ($r > 0.99$) obtained using this method showed that formaldehyde could be determined in the presence of HMPMM at $0^{o}C$, presumably because the N-methylol did not degrade to release formaldehyde under these conditions. At $60^{o}C$ however both formaldehyde and HMPMM were detected by this assay and the colorimetric response was shown to be additive. The method assumes that other formaldehyde precursors behave like HMPMM under the conditions used in this assay. Plasma (0.25 ml) was assayed in identical fashion. Area under the formaldehyde concentration curve X time values were calculated by use of the trapezoidal method.

14.16 In vitro demethylation of HMM and aminopyrine (section 11.3)

Microsomes from the livers of female BDF_1 mice treated with either saline or NMF were prepared as described in 14.10.

Reaction mixtures consisted of microsomal suspension (≡ 0.1g wet liver weight), $MgCl_2$ (5mM), G6P (4 mM), G6P dehydrogenase (15 units ml^{-1}) and NADP (400 μM) in a final volume of Earl's buffer (2.5 ml). Reactions were started by the addition of substrate (0.5 mM) in acetone (0.1 ml), incubated at 37°C in a shaking water bath for 30 min and stopped by the addition of 20% w/v trichloroacetic acid (0.25 ml). Protein was removed by centrifugation and the products of demethylation measured according to the method of Nash.[177]

APPENDICES

Compound Number												
76a	$m/_e$	285	270	208	182	167	153	139	138	124	122	105
	I%	15	3	3	100	68	28	44	35	18	10	15
	$m/_e$	103	97	96	77	76	71					
	I%	34	14	55	23	17	41					
76b	$m/_e$	330	298	283	208	182	167	153	139	138		
	I%	18	56	12	20	97	71	35	44	53		
	$m/_e$	118	96	71								
	I%	38	100	76								
76d	$m/_e$	321	319	306	304	284	268	255	250	248	208	
	I%	3	10	1	3	3	2	2	1	2	4	
	$m/_e$	182	167	153	139	138	124	96	71			
	I%	100	83	31	57	43	18	63	36			
76e	$m/_e$	321	319	306	304	278	277	276	275	250	248	
	I%	3	10	1	3	2	2	5	5	1	3	
	$m/_e$	208	182	167	153	139	138	137	124	111	96	71
	I%	6	100	78	36	67	49	26	22	16	68	44
76f	$m/_e$	321	319	306	304	278	277	276	275	250	248	
	I%	6	18	2	5	2	2	5	5	2	5	
	$m/_e$	208	182	167	153	139	138	137	124	111	96	71
	I%	7	100	89	57	43	50	52	59	29	7	46

Appendix 5.1 Mass spectra of 2-substituted benzylidenehydrazino-4,6-bis(dimethylamino)-s-triazines.

Compound Number															
77a	$m/_e$	283	268	254	240	214	213	180	170	137	103	96	82	67	
	I%	100	31	14	16	10	9	8	13	23	16	19	43	62	
77c	$m/_e$	319	317	304	302	290	288	282	276	274	250	248	137	96	82	67
	I%	30	100	8	23	3	8	15	3	8	3	8	25	22	45	56
77d	$m/_e$	319	317	304	302	290	288	276	274	250	248	137	96	82	69	67
	I%	38	100	12	38	6	18	6	18	3	12	68	44	97	38	100
77b	$m/_e$	328	298	296	253	238	210	182	166	134	96					
	I%	22	10	6	37	27	22	22	30	24	100					

Appendix 5.2 Mass spectra of 3-aryl-s-triazolo[4,3-a]-1,3,5-triazines

Compound Number																	
78a	m/e	283	268	254	240	225	214	180	137	109	96	71	67				
	I%	100	48	25	25	7	20	15	30	18	25	18	29				
78c	m/e	319	317	304	302	290	288	276	274	250	248	180	137	109	96	71	67
	I%	36	100	19	56	11	31	8	28	11	31	42	61	36	64	42	61
78d	m/e	319	317	304	302	290	288	276	274	250	248	180	137	109	96	71	67
	I%	36	100	21	61	9	30	9	27	6	21	24	61	30	52	36	58
78e	m/e	319	317	304	302	290	288	276	274	250	248	180	137	109	96	71	67
	I%	34	100	21	55	11	32	10	27	6	21	24	47	26	44	29	47
78b	m/e	328	313	311	298	285	283	180	137	96	71	69	67				
	I%	100	38	36	36	16	13	56	51	73	36	33	60				
78f	m/e	298	283	268	254	184	180	138	137	113	96	83	71	69	67		
	I%	100	22	16	16	18	19	24	27	21	21	55	21	18	37		
78g	m/e	324	298	283	268	255	254	240	166	149	96	71	69	67			
	I%	9	54	37	15	10	11	10	41	47	100	51	38	24			

Appendix 5.3 Mass spectra of 2-aryl-s-triazolo [2,3-a]-1,3,5-triazines

Day Post implant	Mean tumour volumes (cm^3 ± s.d.) implantation of tumour cells				
	10^6	10^5	10^4	10^3	10^2
12	0.76 ± 0.14				
14	1.35 ± 0.16				
16	2.07 ± 0.24	NM*			
18	2.61 ± 0.28	0.37 ± 0.07			
20	3.36 ± 0.38	0.53 ± 0.13			
22	3.85 ± 0.40	0.82 ± 0.29			
24	4.69 ± 0.74	1.20 ± 0.25	NM		
26	5.78 ± 0.78	1.89 ± 0.37	0.46 ± 0.18		
28	6.52 ± 1.13	2.48 ± 0.74	0.62 ± 0.21	NM	
30	8.03 ± 1.47	2.97 ± 0.83	0.80 ± 0.31	0.29 ± 0.19	
32		3.30 ± 0.65	1.37 ± 0.20	0.38 ± 0.30	
34		4.18 ± 1.10	1.57 ± 0.55	0.74 ± 0.25	
36		4.88 ± 1.05	2.54 ± 0.92	0.98 ± 0.46	
38		5.44 ± 1.89	2.63 ± 1.06	0.97 ± 0.58	
40		6.41 ± 1.97	3.33 ± 0.69	1.58 ± 0.91	0.47 ± 0.41
42		7.01 ± 2.42	3.75 ± 0.79	1.80 ± 0.81	0.73 ± 0.46
44		7.99 ± 1.63	4.44 ± 1.12	2.22 ± 1.18	0.97 ± 0.60
46			4.98 ± 1.30	2.81 ± 1.25	1.68 ± 0.21
48			5.64 ± 1.35	3.69 ± 1.40	1.96 ± 0.63
50			6.18 ± 1.84	4.25 ± 1.19	2.41 ± 0.63
52			6.30 ± 1.55	4.96 ± 1.08	2.83 ± 0.81

* NM = Non-measurable

Appendix 6.1 Mean tumour volumes of the M5 sarcoma after implantation (i.m.) of differing numbers of tumour cells in BDF_1 ♀ mice.

COMPOUND	SCHEDULE	Dose mg kg^{-1}	No. Dead / Total	Mean Body Weight change (g)	MEAN TUMOUR VOLUME (cm^3 ± s.d.) measured on days post implant				$\frac{T}{C}$ x 100%			
					DAY 12	DAY 16	DAY 20	DAY 24	DAY 12	DAY 16	DAY 20	DAY 24
CYCLOPHOSPHAMIDE	Day 1 only	400	$^1/_5$	+0.1	NM	NM	NM	NM				≤4
		200	$^0/_5$	+2.5	NM	NM	NM	0.24 ±0.08			≤6	8
		100	$^0/_5$	+2.7	NM	NM	NM	0.30 ±0.01			≤6	10
		50	$^0/_5$	+3.5	0.29 ±0.10	0.82±0.17	1.74±0.20	2.84 ±0.38	69	75	89	101
		25	$^0/_5$	+3.6	0.34±0.15	0.90±0.18	2.02 ±0.42	3.17 ±0.85	81	82	104	112
		Control	$^0/_{16}$	+2.7	0.42±0.06	1.10±0.28	1.95 ±0.31	2.82 ±0.60	100	100	100	100
	LD_{10} = 370 mg kg^{-1}				LD_{50} = 520 mg kg^{-1}				ID_{90} = 100 mg kg^{-1}			
CHLORAMBUCIL	Day 1 only	100	$^5/_5$	-	‡	‡	‡	‡	-	-	-	-
		50	$^3/_5$	+1.2	NM	NM	0.44 ±0.35	0.89 ±0.16	-	≤11	23	32
		25	$^0/_5$	+2.3	NM	0.42 ±0.09	0.79 ±0.23	1.66 ±0.48	≤30	38	41	59
		12.5	$^0/_5$	+2.5	NM	0.46 ±0.06	0.86 ±0.10	1.88 ±0.38	≤30	42	44	67
		6.25	$^0/_5$	+2.3	0.35 ±0.08	0.69 ±0.07	1.64 ±0.11	2.49 ±0.23	83	63	84	88
		Control	$^0/_{16}$	+2.7	0.42 ±0.06	1.10 ±0.28	1.95 ±0.31	2.82 ±0.60	100	100	100	100
	LD_{10} = 26 mg kg^{-1}				LD_{50} = 45 mg kg^{-1}				(ID_{90} ~ 97 mg kg^{-1})			

‡ = All toxic deaths NM = Non measurable

Appendix 6.2 Chemosensitivity of the M5 sarcoma

COMPOUND	SCHEDULE	Dose mg kg^{-1} injection^{-1}	No. Dead / Total	Mean Body Weight change (g)	MEAN TUMOUR VOLUME (cm^3 ± s.d.) measured on days post implant				$\frac{T}{C}$ x 100%			
					DAY 12	DAY 16	DAY 29	DAY 24	DAY 12	DAY 16	DAY 20	DAY 24
CDDP	Days 1,5,9,13, 17	8	$^4/_5$	- 4.3	NM	NM	NM	NM	-	-	-	≤4
		6	$^0/_5$	- 1.0	NM	NM	NM	NM	-	-	-	≤4
		4	$^0/_5$	+ 0.1	NM	NM	NM	NM	-	-	-	≤4
		2	$^0/_5$	+ 2.9	0.20 ±0.04	0.47 ±0.12	0.85 ±0.18	1.34 ±0.29	44	45	37	42
		1	$^0/_5$	+ 1.9	0.21 ±0.01	0.58 ±0.08	1.21 ±0.15	1.87 ±0.35	47	55	53	58
		Control	$^0/_{19}$	+ 1.9	0.45 ±0.11	1.05 ±0.23	2.30 ±0.45	3.20 ±0.54	100	100	100	100
	LD_{10} = 6.3 mg kg^{-1} 4 days^{-1}				LD_{50} = 7.3 mg kg^{-1} 4 days^{-1}				ID_{90} = 3.6 mg kg^{-1} 4 days^{-1}			
CCNU	Day 1 only	80	$^5/_5$	-	NM	NM	NM	NM	‡	‡	‡	‡
		40	$^0/_5$	+ 0.4	NM	NM	NM	NM	-	-	-	≤4
		20	$^0/_5$	+ 0.6	NM	NM	NM	NM	-	-	-	≤4
		10	$^0/_5$	+ 1.1	NM	NM	0.34 ±0.29	0.79 ±0.46	-	≤11	17	28
		5	$^0/_5$	+ 2.0	0.16 ±0.07	0.42 ±0.21	0.88 ±0.38	1.76 ±0.37	38	38	45	62
		Control	$^0/_{16}$	+ 2.7	0.42 ±0.06	1.10 ±0.28	1.95 ±0.31	2.82 ±0.60	100	100	100	100
	LD_{10} = 43 mg kg^{-1} day^{-1}				LD_{50} = 57 mg kg^{-1} day^{-1}				ID_{90} = 17 mg kg^{-1} day^{-1}			

NM = Non measurable ‡ = All toxic deaths

APPENDIX 6.2 Chemosensitivity of the M5 sarcoma (cont.)

COMPOUND	SCHEDULE	Dose mg kg^{-1} $injection^{-1}$	No. Dead / Total	Mean Body Weight change (g)	MEAN TUMOUR VOLUME (cm^3 ± s.d.) measured on days post implant				$\frac{T}{C}$ X 100%			
					DAY 12	DAY 16	DAY 20	DAY 24	DAY 12	DAY 16	DAY 20	DAY 24
METHOTREXATE	Days 1 - 17	4.0	$^2/_5$	-0.9	0.44 ±0.03	1.14 ±0.29	1.89 ±0.70	3.01 ±1.09	105	104	97	107
		2.0	$^1/_5$	+1.8	0.44 ±0.11	1.05 ±0.22	2.47 ±0.61	3.74 ±0.56	105	95	127	133
		1.0	$^0/_5$	+2.1	0.47 ±0.12	1.23 ±0.34	2.33 ±0.51	3.78 ±0.90	112	112	120	134
		0.5	$^0/_5$	+1.5	0.45 ±0.07	1.04 ±0.29	2.33 ±0.35	3.70 ±0.53	107	95	120	131
		0.25	$^0/_5$	+3.1	0.49 ±0.08	1.10 ±0.15	2.38 ±0.57	3.60 ±0.53	117	100	122	128
		Control	$^0/_{16}$	+2.7	0.42 ±0.06	1.10 ±0.28	1.95 ±0.31	2.82 ±0.60	100	100	100	100
	LD_{10} = 2.6 mg $kg^{-1}day^{-1}$ LD_{50} = 3.8 mg $kg^{-1}day^{-1}$											
5-FLUOROURACIL	Days 1 - 17	40	$^5/_5$	-	‡	‡	‡	‡	-	-	-	-
		20	$^5/_5$	-	‡	‡	‡	‡	-	-	-	-
		10	$^0/_5$	+2.5	0.35 ±0.05	1.05 ±0.27	2.11 ±0.49	3.21 ±0.70	103	94	93	98
		Control	$^0/_{17}$	+1.9	0.34 ±0.08	1.12 ±0.26	2.26 ±0.55	3.28 ±0.74	100	100	100	100
	LD_{10} = 10.7 mg $kg^{-1}day^{-1}$ LD_{50} = 14.2 mg $kg^{-1}day^{-1}$											

‡ = All toxic deaths

Appendix 6.2 Chemosensitivity of the M5 sarcoma (cont.)

COMPOUND	SCHEDULE	Dose mg kg^{-1} injection^{-1}	No. Dead / Total	Mean Body Weight change (g)	MEAN TUMOUR VOLUME (cm^3 ± s.d.) measured on days post implant				$\frac{T}{C}$ x 100%			
					DAY 12	DAY 16	DAY 20	DAY 24	DAY 12	DAY 16	DAY 20	DAY 24
ADRIAMYCIN	Days 1,5,9,13, 17	5.0	$^2/_5$	-0.5	0.16 ±0.07	0.38 ±0.08	0.73 ±0.14	1.01 ±0.34	38	35	37	36
		2.5	$^0/_5$	+1.4	0.33 ±0.05	0.72 ±0.05	1.58 ±0.14	1.96 ±0.49	79	65	81	70
		1.25	$^0/_5$	+1.3	0.34 ±0.07	0.82 ±0.17	1.57 ±0.26	2.38 ±0.66	81	75	81	84
		Control	$^0/_{16}$	+2.7	0.42 ±0.06	1.10 ±0.28	1.95 ±0.31	2.82 ±0.60	100	100	100	100
	LD_{10} = 3.7 mg kg^{-1}4 days^{-1}				LD_{50} = 5.6 mg kg^{-1}4 days^{-1}							
DTIC	Days 1,5,9,13, 17	400	$^5/_5$	-	‡	‡	‡	‡	-	-	-	-
		200	$^1/_5$	-0.7	NM	NM	NM	NM	-	-	-	≤4
		100	$^0/_5$	+0.8	NM	NM	NM	NM	-	-	-	≤4
		50	$^0/_5$	+0.7	NM	NM	NM	NM	-	-	-	≤4
		Control	$^0/_{16}$	+2.7	0.42 ±0.06	1.10 ±0.28	1.95 ±0.31	2.82 ±0.60	100	100	100	100
	Days 1,5,9,13 17	25	$^0/_5$	+2.1	NM	0.22 ±0.07	0.32 ±0.12	0.43 ±0.11	≤ 28	21	14	13
		12.5	$^0/_5$	+1.2	NM	0.42 ±0.04	0.82 ±0.11	1.18 ±0.13	≤ 28	40	36	37
		6.25	$^0/_5$	+2.0	0.36 ±0.11	0.79 ±0.22	1.58 ±0.11	2.33 ±0.41	80	75	69	73
		Control	$^0/_{19}$	+1.9	0.45 ±0.11	1.05 ±0.23	2.30 ±0.45	3.20 ±0.54	100	100	100	100
	LD_{10} = 185 mg kg^{-1}4 days^{-1}				LD_{50} = 260 mg kg^{-1}4 days^{-1}				ID_{90} = 32 mgkg^{-1}4 days^{-1}			

‡ = All toxic deaths NM = Non measurable

Appendix 6.2 Chemosensitivity of the M5 sarcoma (cont.)

COMPOUND	SCHEDULE	Dose mg kg^{-1} injection^{-1}	No. Dead / Total	Mean Body weight change (g)	MEAN TUMOUR VOLUME (cm^3 ± s.d.) measured on days post implant				$\frac{T}{C}$ X 100%			
					DAY 12	DAY 16	DAY 20	DAY 24	DAY 12	DAY 16	DAY 20	DAY 24
PROCARBAZINE	Days 1,5,9,13, 17	100	0/5	-0.6	NM	NM	NM	NM	-	-	-	≤4
		50	0/5	+0.4	NM	NM	NM	NM	-	-	-	≤4
		25	0/5	+1.4	NM	NM	NM	0.25 ±0.13	-	-	≤6	9
		12.5	0/5	+2.1	NM	0.26 ±0.12	0.37 ±0.08	0.70 ±0.11	≤30	24	19	25
		Control	0/16	+2.7	0.42 ±0.06	1.10 ±0.15	1.95 ±0.31	2.82 ±0.60	100	100	100	100
	LD_{10} = 240 mg kg^{-1} 4 days^{-1}				LD_{50} = 470 mg kg^{-1}4days^{-1}				ID_{90} = 23 mg kg^{-1} 4 days^{-1}			
TREOSULPHAN	Days 1 - 17	800	1/5	-1.9	NM	NM	0.23 ±0.15	0.28 ±0.09	-	≤9	9	6
		Control	0/16	+2.3	0.44 ±0.07	1.34 ±0.23	2.65 ±0.33	4.36 ±0.58	100	100	100	100
	Days 1 - 17	400	0/5	+0.4	NM	NM	0.17 ±0.09	0.33 ±0.22	-	≤18	10	13
		200	0/5	+2.9	0.15 ±0.05	0.19 ±0.15	0.60 ±0.35	1.21 ±0.45	47	27	35	46
		100	0/5	+2.6	0.21 ±0.11	0.24 ±0.16	1.00 ±0.30	1.88 ±0.46	66	34	59	72
		50	0/5	+3.0	0.21 ±0.11	0.61 ±0.20	1.88 ±0.42	2.71 ±0.50	66	87	111	103
		Control	0/18	+3.0	0.32 ±0.13	0.70 ±0.24	1.70 ±0.44	2.62 ±0.65	100	100	100	100
	LD_{10} = 570 mg kg^{-1}day^{-1}				LD_{50} > 800 mg kg^{-1}day^{-1}				ID_{90} ~ 420 mg kg^{-1}day^{-1}			

NM = Non measurable

Appendix 6.2 Chemosensitivity of the M5 sarcoma (cont.)

COMPOUND	SCHEDULE	Dose mg kg^{-1} injection^{-1}	No. Dead / Total	Mean Body Weight Change (g)	MEAN TUMOUR VOLUME ($cm^3 \pm$ s.d.) measured on days post implant				$\frac{T}{C}$ X 100%			
					DAY 12	DAY 16	DAY 20	DAY 24	DAY 12	DAY 16	DAY 20	DAY 24
HEXAMETHYLMELAMINE	days 1 - 17 A	80	$^1/_{10}$	-1.2	NM	0.13 ±0.02	0.30 ±0.11	0.75 ±0.20	≤23	12	16	26
		60	$^0/_{10}$	-0.4	NM	0.20 ±0.09	0.58 ±0.08	1.35 ±0.27	≤23	19	32	46
		Control	$^0/_{27}$	+1.0	0.55 ±0.12	1.08 ±0.22	1.84 ±0.39	2.92 ±0.68	100	100	100	100
	days 1,3,5,7,9 11,13,15, 17 B	150	$^1/_{10}$	-0.4	NM	0.19 ±0.06	0.34 ±0.14	0.87 ±0.31	≤23	18	18	30
		100	$^0/_{10}$	+0.3	0.15 ±0.04	0.40 ±0.17	0.66 ±0.38	1.36 ±0.40	27	37	36	47
		Control	$^0/_{20}$	+1.0	0.55 ±0.12	1.08 ±0.22	1.84 ±0.39	2.92 ±0.68	100	100	100	100
	1,3,5,7, 9,11 C	250	$^6/_{10}$	-2.6	NM	0.22 ±0.10	0.29 ±0.10	0.65 ±0.17	≤22	19	14	19
		200	$^2/_{10}$	-1.2	NM	0.31 ±0.17	0.58 ±0.28	1.29 ±0.38	≤22	27	27	38
		150	$^1/_{10}$	-1.1	NM	0.38 ±0.05	0.74 ±0.17	1.47 ±0.49	≤22	33	35	44
		Control	$^0/_{20}$	+1.0	0.58 ±0.30	1.15 ±0.50	2.11 ±0.67	3.37 ±0.69	100	100	100	100
	days 1,5,9 8 times per day D	50	$^{10}/_{10}$	-3.9	ǂ	ǂ	ǂ	ǂ	-	-	-	-
		40	$^6/_{10}$	-2.2	NM	0.15 ±0.06	0.25 ±0.09	0.47 ±0.13	≤24	13	14	17
		30	$^3/_{10}$	-1.9	0.14 ±0.04	0.26 ±0.08	0.47 ±0.16	1.22 ±0.50	26	23	27	45
		Control	$^0/_{20}$	+0.7	0.53 ±0.14	1.14 ±0.26	1.75 ±0.40	2.73 ±0.52	100	100	100	100

ǂ = All toxic deaths NM = Non measurable

Appendix 7.1 a) Comparison of chronic schedules for hexamethylmelamine against the M5 sarcoma.

COMPOUND	SCHEDULE	Dose mg kg^{-1} $injection^{-1}$	No. Dead / Total	Mean Body Weight Change (g)	MEAN TUMOUR VOLUME (cm^3 ± s.d.) measured on days post implant				$\frac{T}{C}$ X 100%			
					DAY 12	DAY 16	DAY 20	DAY 24	DAY 12	DAY 16	DAY 20	DAY 24
PENTAMETHYLMELAMINE	days 1 - 17 A	75	1/10	-1.1	NM	0.20 ±0.08	0.53 ±0.12	1.18 ±0.22	≤23	19	29	40
		56	1/10	-0.8	NM	0.22 ±0.09	0.59 ±0.13	1.32 ±0.23	≤23	20	32	45
		Control	0/20	+1.0	0.55 ±0.22	1.08 ±0.39	1.84 ±0.39	2.92 ±0.68	100	100	100	100
	1,3,5,7, 9,11,13, 15,17 B	140	0/10	0	0.16 ±0.06	0.26 ±0.10	0.58 ±0.12	1.01 ±0.18	29	24	32	35
		94	0/10	-0.4	0.15 ±0.06	0.40 ±0.10	0.86 ±0.16	1.49 ±0.24	27	37	47	51
		Control	0/20	+1.0	0.55 ±0.12	1.08 ±0.22	1.84 ±0.39	2.92 ±0.68	100	100	100	100
	1,3,5,7, 9,11 C	234	6/10	-1.6	NM	0.34 ±0.11	0.53 ±0.11	1.08 ±0.32	≤22	30	25	32
		187	2/10	+0.1	NM	0.41 ±0.11	0.59 ±0.17	1.08 ±0.34	≤22	36	28	32
		140	0/10	+1.1	NM	0.66 ±0.30	0.91 ±0.20	1.65 ±0.47	≤22	57	43	49
		Control	0/20	+1.0	0.58 ±0.30	1.15 ±0.05	2.11 ±0.67	3.37 ±0.69	100	100	100	100

Appendix 7.1 b) Comparison of chronic schedules for pentamethylmelamine against the M5 sarcoma

COMPOUND	SCHEDULE	Dose mg kg^{-1} $injection^{-1}$	No. Dead / Total	Mean Body Weight change (g)	MEAN TUMOUR VOLUME (cm^3 ± s.d.) measured on days post implant				$\frac{T}{C}$ x 100%			
					DAY 12	DAY 16	DAY 20	DAY 24	DAY 12	DAY 16	DAY 20	DAY 24
N-HYDROXYMETHYLPENTAMETHYLMELAMINE	days 1 - 17 A	86	0/10	-1.6	NM	0.14 ±0.03	0.40 ±0.12	0.85 ±0.31	⩽23	13	22	29
		65	0/10	-0.4	NM	0.20 ±0.09	0.62 ±0.11	1.29 ±0.26	⩽23	19	34	44
		Control	0/20	+0.1	0.55 ±0.12	1.08 ±0.22	1.84 ±0.39	2.92 ±0.68	100	100	100	100
	1,3,5,7, 9,11,13, 15,17 B	161	0/10	-1.1	NM	0.20 ±0.09	0.49 ±0.19	0.97 ±0.29	⩽23	19	27	33
		108	0/10	+0.1	0.14 ±0.04	0.36 ±0.19	0.80 ±0.25	1.60 ±0.43	25	33	43	55
		Control	0/20	+1.0	0.55 ±0.12	1.08 ±0.22	1.84 ±0.39	2.92 ±0.68	100	100	100	100
	1,3,5,7, 9,11 C	270	5/10	-1.8	NM	0.19 ±0.22	0.50 ±0.18	0.98 ±0.68	⩽22	17	24	29
		215	2/10	-2.1	NM	0.32 ±0.07	0.62 ±0.21	1.13 ±0.36	⩽22	28	29	34
		161	2/10	-1.0	NM	0.44 ±0.16	0.91 ±0.39	1.58 ±0.41	⩽22	38	43	47
		Control	0/20	+1.0	0.58 ±0.30	1.15 ±0.50	2.11 ±0.67	3.37 ±0.69	100	100	100	100

NM + Non measurable

Appendix 7.1.c) Comparison of chronic schedules for N-hydroxymethylpentamethylmelamine against the M5 sarcoma

COMPOUND	SCHEDULE	Dose mg kg^{-1} day^{-1}	No. Dead / Total	Mean Body Weight change (g)	MEAN TUMOUR VOLUME (cm^3 ± s.d.) measured on days post implant				$\frac{T}{C}$ x 100%			
					DAY 12	DAY 16	DAY 20	DAY 24	DAY 12	DAY 16	DAY 20	DAY 24
HEXAMETHYLMELAMINE	days 1 - 17	150	5/5	-	-	-	-	-	-	-	-	-
		125	5/5	-	-	-	-	-	-	-	-	-
		100	1/8	-0.7	NM	NM	NM	NM	-	-	-	⩽5
		75	0/8	+0.7	NM	NM	NM	0.25 ±0.07	-	-	⩽7	9
		50	0/8	+1.4	NM	NM	0.26 ±0.18	0.62 ±0.33	-	⩽13	14	23
		25	0/8	+2.6	0.16 ±0.04	0.32 ±0.12	0.87 ±0.21	1.57 ±0.32	48	34	47	58
		Control	0/18	+2.6	0.33 ±0.05	0.94 ±0.15	1.86 ±0.26	2.72 ±0.48	100	100	100	100
	LD_{10} = 99 mg kg^{-1}day^{-1}				LD_{50} = 110 mg kg^{-1}day^{-1}				ID_{90} = 69 mg kg^{-1}day^{-1}			
PENTAMETHYLMELAMINE	days 1 - 17	200	5/5	-	-	-	-	-	-	-	-	-
		160	2/5	-4.1	NM	NM	NM	0.19 ±0.12	-	-	⩽5	4
		100	0/5	-2.5	NM	NM	0.17 ±0.10	0.82 ±0.29	-	⩽9	6	19
		80	0/5	-0.2	NM	NM	0.52 ±0.15	1.39 ±0.60	-	⩽9	20	32
		Control	0/16	+2.5	0.44 ±0.07	1.34 ±0.23	2.65 ±0.33	4.36 ±0.51	100	100	100	100
	LD_{10} = 144 mg kg^{-1}day^{-1}				LD_{50} = 167 mg kg^{-1}day^{-1}				ID_{90} = 132 mg kg^{-1}day^{-1}			

NM = Non measurable

Appendix 7.2 Activity of the alkylmelamines against the M5 sarcoma

COMPOUND	SCHEDULE	Dose mg $kg^{-1}day^{-1}$	No. Dead / Total	Mean Body Weight Change (g)	MEAN TUMOUR VOLUME ($cm^3 \pm$ s.d.) measured on days post implant				$\frac{T}{C}$ x 100%			
					DAY 12	DAY 16	DAY 20	DAY 24	DAY 12	DAY 16	DAY 20	DAY 24
2,2,4,4-TETRAMETHYLMELAMINE	days 1 - 17	200	4/5	-5.9	-	-	-	-	-	-	-	-
		160	1/5	+1.6	NM	NM	NM	0.22 ±0.18	-	-	⩽7	8
		80	0/5	+3.4	NM	NM	0.35 ±0.14	0.83 ±0.36	-	⩽18	21	32
		40	0/5	+3.7	NM	NM	0.71 ±0.13	1.22 ±0.51	-	⩽18	42	47
		20	0/5	+3.6	0.25 ±0.11	0.44 ±0.28	1.44 ±0.86	2.12 ±1.00	76	63	85	81
		10	0/5	+3.9	0.29 ±0.22	0.50 ±0.25	1.54 ±0.40	2.34 ±0.47	88	71	91	89
		Control	0/18	+3.0	0.33 ±0.13	0.70 ±0.24	1.70 ±0.44	2.62 ±0.65	100	100	100	100
	LD_{10} = 154 mg $kg^{-1}day^{-1}$				LD_{50} = 180 mg $kg^{-1}day^{-1}$				ID_{90} = 155 mg $kg^{-1}day^{-1}$			
2,4,6-TRIMETHYLMELAMINE	days 1 - 17	640	5/5	-	-	-	-	-	-	-	-	-
		320	5/5	-	-	-	-	-	-	-	-	-
		160	0/5	+1.2	0.56 ±0.18	1.38 ±0.26	2.18 ±0.57	3.69 ±0.88	67	71	69	80
		Control	0/17	+2.4	0.84 ±0.20	1.94 ±0.43	3.16 ±0.63	4.61 ±0.83	100	100	100	100
	LD_{10} = 170 mg $kg^{-1}day^{-1}$				LD_{50} = 225 mg $kg^{-1}day^{-1}$							

NM = Non measurable

Appendix 7.2 Activity of the alkylmelamines against the M5 sarcoma (cont.)

COMPOUND	SCHEDULE	Dose mg $kg^{-1}day^{-1}$	No. Dead / Total	Mean Body Weight Change (g)	MEAN TUMOUR VOLUME ($cm^3 \pm$ s.d.) measured on days post implant				$\frac{T}{C}$ x 100%			
					DAY 12	DAY 16	DAY 20	DAY 24	DAY 12	DAY 16	DAY 20	DAY 24
MELAMINE	days 1 - 17	1500	4/5	+0.3	NM	0.67	1.45	2.74	≤28	64	63	86
		1000	1/5	+3.6	0.25 ±0.10	0.72 ±0.11	1.83 ±0.17	2.43 ±0.19	56	69	80	76
		Control	0/19	+1.9	0.45 ±0.11	1.05 ±0.23	2.30 ±0.45	3.20 ±0.54	100	100	100	100
	days 1 - 17	750	0/5	+2.1	0.40 ±0.11	1.11 ±0.34	1.83 ±0.11	2.91 ±0.66	103	113	103	105
		500	0/5	+1.2	0.38 ±0.12	0.83 ±0.22	1.69 ±0.23	2.74 ±0.30	97	85	95	99
		250	0/5	+2.0	0.37 ±0.13	1.04 ±0.24	1.84 ±0.15	2.71 ±0.20	95	106	103	98
		Control	0/10	+2.5	0.39 ±0.10	0.98 ±0.27	1.78 ±0.19	2.76 ±0.30	100	100	100	100
	LD_{10} = 960 mg $kg^{-1}day^{-1}$				LD_{50} = 1180 mg $kg^{-1}day^{-1}$							
HEXAETHYLMELAMINE	days 1 - 17	1000	1/5	+1.0	NM	0.66 ±0.03	1.76 ±0.36	2.26 ±0.64	≤ 28	63	77	71
		Control	0/19	+1.9	0.45 ±0.11	1.05 ±0.23	2.30 ±0.45	3.20 ±0.54	100	100	100	100
	days 1 - 17	750	1/5	+2.3	0.25 ±0.13	0.66 ±0.27	1.13 ±0.37	1.67 ±0.60	64	67	63	61
		500	0/5	+1.8	0.34 ±0.10	0.67 ±0.16	1.36 ±0.47	2.31 ±0.89	87	68	76	84
		250	0/5	+2.9	0.34 ±0.07	0.76 ±0.18	1.54 ±0.26	2.69 ±0.48	87	78	87	97
		Control	0/10	+2.5	0.39 ±0.10	0.98 ±0.27	1.78 ±0.19	2.76 ±0.30	100	100	100	100
	LD_{10} ~ 820 mg $kg^{-1}day^{-1}$											

NM = Non measurable

Appendix 7.2 Activity of the alkylmelamines against the M5 sarcoma (cont.)

COMPOUND	Schedule days of injection	Dose $mgkg^{-1}$ day^{-1}	Mean body weight change (g)	No. dead / Total	Mean tumour volume (cm^3 ± s.d.) measured on days post implant				$\frac{T}{C}$ X 100%			
					DAY 7	DAY 10	DAY 14	DAY 19	DAY 7	DAY 10	DAY 14	DAY 19
HMM	1 - 9	80	-1.0	0/10	0.22 ±0.07	0.36 ±0.17	0.81 ±0.38	1.73 ±0.53	37	25	35	40
TriMM	1 - 9	150	-1.1	0/10	0.49 ±0.20	1.11 ±0.42	1.98 ±0.53	3.33 ±0.45	83	78	85	77
Control			+1.2	0/40	0.59 ±0.12	1.43 ±0.30	2.33 ±0.40	4.30 ±0.77	100	100	100	100

Appendix 7.3 Activity of alkylmelamines against the Sarcoma 180

COMPOUND	SCHEDULE	Dose mg $kg^{-1}day^{-1}$	No. Dead / Total	Mean Body Weight Change (g)	MEAN TUMOUR VOLUME ($cm^3 \pm$ s.d.) measured on days post implant				$\frac{T}{C}$ X 100%			
					DAY 12	DAY 16	DAY 20	DAY 24	DAY 12	DAY 16	DAY 20	DAY 24
N-HYDROXY PENTAMETHYLMELAMINE	days 1 - 17	640	$^4/_5$	-	-	-	-	-	-	-	-	-
		320	$^0/_5$	-0.6	0.53 ±0.10	1.38 ±0.10	2.13 ±0.24	3.35 ±0.14	63	71	67	73
		160	$^0/_5$	+2.5	0.59 ±0.11	1.37 ±0.32	2.45 ±0.68	3.63 ±0.88	70	71	78	79
		80	$^0/_5$	+1.9	0.63 ±0.09	1.67 ±0.19	2.57 ±0.19	3.53 ±0.56	75	86	81	77
		40	$^0/_5$	+1.2	0.64 ±0.23	1.69 ±0.54	2.84 ±0.89	4.17 ±1.15	76	87	90	90
		20	$^0/_5$	+0.3	0.81 ±0.11	2.05 ±0.22	3.08 ±0.30	4.27 ±0.50	96	106	97	93
		Control	$^0/_{17}$	+2.4	0.84 ±0.20	1.94 ±0.43	3.16 ±0.63	4.61 ±0.83	100	100	100	100
	LD_{10} = 350 mg $kg^{-1}day^{-1}$ LD_{50} = 494 mg $kg^{-1}day^{-1}$											
$N^2,N^{2'}$-METHYLENE BIS PENTAMETHYLMELAMINE	days 1 - 17	640	$^1/_5$	-	NM	0.51 ±0.45	1.26 ±0.83	1.90 ±1.11	⩽15	26	40	41
		320	$^0/_5$	+1.9	0.62 ±0.12	1.14 ±0.40	2.19 ±0.36	2.98 ±0.69	74	59	69	65
		160	$^0/_5$	+2.4	0.64 ±0.05	1.65 ±0.44	2.73 ±0.41	3.58 ±0.30	76	85	86	78
		80	$^0/_5$	+2.5	0.83 ±0.20	2.07 ±0.38	3.42 ±0.76	4.76 ±0.86	99	107	108	103
		Control	$^0/_{17}$	+2.4	0.84 ±0.20	1.94 ±0.43	3.16 ±0.63	4.61 ±0.83	100	100	100	100
	$LD_{10} \sim$ 450 mg $kg^{-1}day^{-1}$ $ID_{90} \geqslant$ 640 mg $kg^{-1}day^{-1}$											

NM = Non measurable

Appendix 7.4 The activity of N-substituted-PMM compounds against the M5 sarcoma.

COMPOUND	SCHEDULE	Dose mg $kg^{-1}day^{-1}$	No. Dead / Total	Mean Body Weight change (g)	MEAN TUMOUR VOLUME ($cm^3 \pm$ s.d.) measured on days post implant				$\frac{T}{C}$ X 100%			
					DAY 12	DAY 16	DAY 20	DAY 24	DAY 12	DAY 16	DAY 20	DAY 24
N-HYDROXYMETHYL-PENTAMETHYLMELAMINE	days 1 - 17	320	5/5	-	-	-	-	-	-	-	-	-
		160	5/5	-	-	-	-	-	-	-	-	-
		80	0/5	-1.8	NM	0.17 ±0.10	0.59 ±0.15	1.16 ±0.35	⩽15	9	19	25
		40	0/5	+0.6	0.39 ±0.17	1.15 ±0.23	1.64 ±0.59	2.75 ±1.05	46	59	52	60
		Control	0/17	+2.4	0.84 ±0.20	1.94 ±0.43	3.16 ±0.63	4.61 ±0.83	100	100	100	100
		LD_{10} = 86 mg $kg^{-1}day^{-1}$			LD_{50} = 114 mg $kg^{-1}day^{-1}$				ID_{90} = 110 mg $kg^{-1}day^{-1}$			
N-FORMYL PENTAMETHYLMELAMINE	days 1 - 17	320	5/5	-	-	-	-	-	-	-	-	-
		160	2/5	0	NM	NM	0.72 ±0.21	1.51 ±0.36	—	⩽6	23	33
		Control	0/17	+2.4	0.84 ±0.20	1.94 ±0.43	3.16 ±0.63	4.61 ±0.83	100	100	100	100
	days 1 - 17	80	0/5	+1.9	NM	0.28 ±0.12	0.65 ±0.16	1.53 ±0.27	⩽37	25	29	47
		40	0/5	+2.7	0.17 ±0.09	0.85 ±0.28	1.89 ±0.17	2.98 ±0.51	50	76	84	91
		20	0/5	+3.6	0.28 ±0.05	0.95 ±0.11	1.84 ±0.32	3.14 ±0.60	82	85	81	96
		10	0/5	+3.4	0.36 ±0.06	1.53 ±0.36	2.25 ±0.73	3.39 ±0.73	106	137	100	103
		Control	0/17	+1.9	0.34 ±0.08	1.12 ±0.26	2.26 ±0.55	3.28 ±0.74	100	100	100	100
		LD_{10} = 95 mg $kg^{-1}day^{-1}$			LD_{50} = 187 mg $kg^{-1}day^{-1}$				ID_{90} = 198 mg $kg^{-1}day^{-1}$			

NM = Non measurable

Appendix 7.4 The activity of N-substituted-PMM compounds against the M5 sarcoma (cont.)

COMPOUND	Schedule (days of injection)	Dose $mgkg^{-1}$ day^{-1}	No. dead / Total	Mean body weight change (g)	Mean tumour volume (cm^3 ± s.d.) measured on days post implant				$\frac{T}{C}$ x 100%			
					DAY 12	DAY 16	DAY 20	DAY 24	DAY 12	DAY 16	DAY 20	DAY 24
CBDT	1 - 17	640	5/5	-	-	-	-	-	-	-	-	-
		320	3/5	+2.2	0.89 ±0.06	2.16 ±0.06	3.15 ±0.0	4.09 ±0.35	106	111	100	89
		160	1/5	+0.9	0.83 ±0.11	2.09 ±0.67	3.23 ±0.49	4.44 ±0.83	99	108	102	96
		80	0/5	+2.1	0.75 ±0.24	2.06 ±0.32	3.42 ±0.33	5.30 ±0.70	89	106	108	115
		Control	0/17	+2.4	0.84 ±0.20	1.94 ±0.43	3.16 ±0.63	4.61 ±0.83	100	100	100	100
		LD_{10} = 135 $mgkg^{-1}day^{-1}$					LD_{50} = 270 $mgkg^{-1}day^{-1}$					
HBDT	1 - 17	640	5/5	-	-	-	-	-	-	-	-	-
		320	5/5	-	-	-	-	-	-	-	-	-
		160	1/5	+0.6	0.66 ±0.02	1.72 ±0.31	2.61 ±0.39	3.58 ±0.73	79	89	83	78
		80	0/5	+1.5	0.69 ±0.16	1.84 ±0.27	3.16 ±0.35	4.40 ±0.43	82	95	100	95
		40	0/5	+1.6	0.60 ±0.19	1.73 ±0.18	2.87 ±0.27	4.70 ±0.64	71	89	91	102
		Control	0/17	+2.4	0.84 ±0.20	1.94 ±0.43	3.16 ±0.63	4.61 ±0.83	100	100	100	100
		LD_{10} = 148 $mgkg^{-1}day^{-1}$					LD_{50} = 208 $mgkg^{-1}day^{-1}$					
ABDT	1 - 17	160	5/5	-	-	-	-	-	-	-	-	-
		80	3/5	+1.0	0.85 ±0.12	2.07 ±0.07	3.44 ±0.57	4.70 ±0.10	101	107	109	102
		40	0/5	+2.8	0.74 ±0.19	1.91 ±0.38	3.09 ±0.76	4.62 ±0.66	88	98	98	100
		Control	0/17	+2.4	0.84 ±0.20	1.94 ±0.43	3.16 ±0.63	4.61 ±0.83	100	100	100	100
		LD_{10} = 45 $mgkg^{-1}day^{-1}$					LD_{50} = 72 $mgkg^{-1}day^{-1}$					

Appendix 7.5a Activity of 2-substituted-4,6-bis(dimethylamino)-s-triazines against the M5 sarcoma

COMPOUND	Schedule	Dose $mgkg^{-1}$ day^{-1}	No. dead / Total	Mean body weight change (g)	Mean tumour volume (cm^3 ± s.d.)			$\frac{T}{C}$ X 100%		
					DAY 14	DAY 19	DAY 24	DAY 14	DAY 19	DAY 24
HMM	Days 14 - 19	120	5/5	-4.3	-	-	-	-	-	-
		80	0/5	-2.1	0.91 ±0.22	0.44 ±0.12	NM	103	21	⩽4
		40	0/5	-0.2	0.99 ±0.41	0.77 ±0.32	NM	113	37	⩽4
		20	0/5	+0.8	0.95 ±0.14	1.32 ±0.12	0.98 ±0.51	108	63	28
		10	0/5	+0.7	0.95 ±0.06	1.70 ±0.31	2.89 ±0.27	108	81	81
		5	0/5	+0.9	0.89 ±0.19	2.28 ±0.55	3.84 ±0.70	101	109	108
		Control	0/20	+0.8	0.88 ±0.28	2.10 ±0.39	3.55 ±0.70	100	100	100
	LD_{10} = 84 $mgkg^{-1}day^{-1}$				LD_{50} = 98 $mgkg^{-1}day^{-1}$			ID_{90} = 26 $mgkg^{-1}day^{-1}$		
N-FORMYL PENTAMETHYLMELAMINE	Days 14 - 19	160	5/5	-2.2	2.34 ±0.22	1.01 ±0.17	-	96	24	-
		80	1/5	-0.3	2.35 ±0.31	1.27 ±0.83	NM	97	30	⩽2
		40	0/5	+0.4	2.43 ±0.34	2.62 ±0.91	0.40 ±0.31	100	62	6
		20	0/5	+1.0	2.39 ±0.22	3.17 ±0.29	3.36 ±0.60	98	75	49
		10	0/5	+1.0	2.42 ±0.28	5.99 ±0.61	5.99 ±0.61	100	90	88
		Control	0/7	+1.6	2.43 ±0.25	6.81 ±1.11	6.81 ±1.11	100	100	100
	LD_{10} = 74 $mgkg^{-1}day^{-1}$				LD_{50} = 105 $mgkg^{-1}day^{-1}$			ID_{90} = 38 $mgkg^{-1}day^{-1}$		

Appendix 7.5b Activity of N-substituted PMM compounds against the PC6 plasmacytoma

COMPOUND	SCHEDULE	Dose $mgkg^{-1}$	No. Dead / Total	Mean Body Weight Change (g)	Mean tumour volume (cm^3 ± s.d.) measured on days post implant			$\frac{T}{C}$ X 100%		
					DAY 14	DAY 19	DAY 24	DAY 14	DAY 19	DAY 24
CCNU	DAY 14 only	320	$^5/_5$	-	1.12 ± 0.15	-	-	114	-	-
		160	$^5/_5$	-	0.81 ± 0.26	-	-	83	-	-
		80	$^3/_5$	-5.7	1.04 ± 0.31	NM	NM	106	⩽5	⩽3
		40	$^1/_5$	-2.6	0.79 ± 0.14	NM	NM	81	⩽5	⩽3
		20	$^0/_5$	-0.7	0.97 ± 0.30	0.21 ± 0.12	NM	99	9	⩽3
		10	$^0/_5$	+0.9	0.99 ± 0.28	0.20 ± 0.08	NM	101	8	⩽3
		5	$^0/_5$	+1.9	0.86 ± 0.22	0.25 ± 0.10	NM	88	11	⩽3
		2.5	$^0/_5$	+1.5	0.88 ± 0.24	0.34 ± 0.08	NM	90	14	⩽3
		1.25	$^0/_5$	+1.3	0.76 ± 0.15	0.33 ± 0.07	NM	78	14	⩽3
		0.63	$^0/_5$	+1.4	0.68 ± 0.37	0.53 ± 0.14	NM	70	22	⩽3
		0.31	$^0/_5$	+1.7	0.75 ± 0.15	0.42 ± 0.10	NM	77	18	⩽3
		Control	$^0/_{22}$	+2.4	0.98 ± 0.39	2.37 ± 0.56	3.66 ± 0.62	100	100	100
	DAY 14 only	0.16	$^0/_5$	+3.9	1.13 ± 0.38	3.61 ± 0.29	6.03 ± 0.72	97	96	102
		0.08	$^0/_5$	+3.0	1.06 ± 0.21	3.16 ± 0.28	5.47 ± 0.64	91	84	93
		Control	$^0/_{17}$	+3.5	1.17 ± 0.26	3.27 ± 0.56	5.90 ±1.12	100	100	100
	LD_{10} = 34 mg kg^{-1}				LD_{50} = 67 mg kg^{-1}		ID90 = 0.3 mg kg^{-1}			

Appendix 7.5c Activity of CCNU against the PC6 plasmacytoma

COMPOUND	Schedule	Dose $mgkg^{-1}$	No. Dead / Total	Mean Body weight change (g)	Mean tumour volume ($cm^3 \pm$ s.d.) measured on days post implant			$\frac{T}{C}$ x 100%		
					DAY 14	DAY 19	DAY 24	DAY 14	DAY 19	DAY 24
CYCLOPHOSPHAMIDE	DAY 14 only	640	$^5/_5$	-	1.14±0.45	-	-	116	–	—
		320	$^0/_5$	-3.5	0.89±0.27	NM	NM	91	⩽5	⩽3
		160	$^0/_5$	-1.4	1.01±0.21	NM	NM	103	⩽5	⩽3
		80	$^0/_5$	-0.9	1.01±0.18	NM	NM	103	⩽5	⩽3
		40	$^0/_5$	+0.2	1.03±0.28	0.33±0.13	NM	105	14	⩽3
		20	$^0/_5$	+0.4	1.08±0.25	0.33±0.12	NM	110	14	⩽3
		10	$^0/_5$	+0.8	0.83±0.18	0.34±0.06	NM	85	14	⩽3
		5	$^0/_5$	+1.6	0.90±0.26	0.48±0.15	NM	92	20	⩽3
		2.5	$^0/_5$	+2.0	0.91±0.34	0.59±0.19	0.42±0.27	94	25	11
		1.25	$^0/_5$	+1.5	0.83±0.23	0.92±0.21	1.96±0.58	85	39	54
		0.63	$^0/_5$	+2.9	0.97±0.30	1.57±0.36	2.89±0.67	99	66	80
		0.31	$^0/_5$	+2.9	0.99±0.21	1.68±0.42	3.11±0.57	101	71	85
		Control	$^0/_{22}$	+2.4	0.98±0.39	2.37±0.56	3.66±0.62	100	100	100
		LD_{10} = 343 $mgkg^{-1}day^{-1}$			LD_{50} = 452 $mgkg^{-1}day^{-1}$			ID_{90} = 2.5 $mgkg^{-1}day^{-1}$		

Appendix 7.5c Activity of cyclophosphamide against the PC6 plasmacytoma

COMPOUND	Schedule	Dose $mgkg^{-1}$	No. dead / Total	Mean body weight change (g)	Mean tumour volume ($cm^3 \pm$ s.d.) measured on days post implant			$\frac{T}{C}$ X 100%		
					DAY 14	DAY 19	DAY 24	DAY 14	DAY 19	DAY 24
CHLORAMBUCIL	DAY 14 only	80	$^5/_5$	-	-	-	-	-	-	-
		60	$^0/_5$	-3.2	-	-	-	-	-	-
		40	$^0/_5$	-2.5	0.88 ± 0.24	0.25 ± 0.11	NM	100	12	$\leqslant 4$
		20	$^0/_5$	-1.7	0.79 ± 0.26	0.30 ± 0.01	NM	90	14	$\leqslant 4$
		10	$^0/_5$	-1.3	0.79 ± 0.17	0.43 ± 0.09	NM	90	20	$\leqslant 4$
		5	$^0/_5$	-0.8	0.93 ± 0.20	0.54 ± 0.05	NM	106	26	$\leqslant 4$
		2.5	$^0/_5$	-0.3	0.92 ± 0.26	0.80 ± 0.18	0.50 ± 0.40	105	38	14
		1.25	$^0/_5$	+2.3	0.98 ± 0.33	1.89 ± 0.55	3.57 ± 0.70	111	90	101
		0.625	$^0/_5$	+2.6	0.97 ± 0.39	2.15 ± 0.70	3.25 ± 0.69	110	102	92
		Control	$^0/_5$	+1.9	0.88 ± 0.28	2.10 ± 0.39	3.55 ± 0.70	100	100	100
		LD_{10} = 62 $mgkg^{-1}$			LD_{50} = 70 $mgkg^{-1}$			ID_{90} = 2.6 $mgkg^{-1}$		

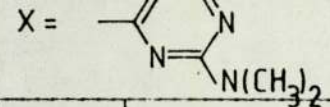
Appendix 7.5c Activity of chlorambucil against the PC6 plasmacytoma

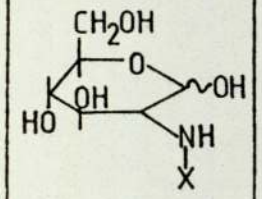
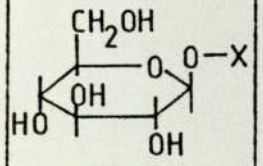
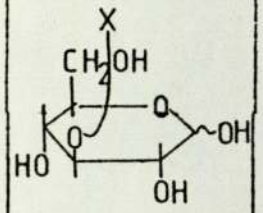
COMPOUND	SCHEDULE	Dose $mgkg^{-1}$	No. Dead / Total	Mean Body Weight Change (g)	Mean tumour volume (cm^3 ± s.d.) measured on day post implant			$\frac{T}{C}$ X 100%		
					DAY 14	DAY 19	DAY 24	DAY 14	DAY 19	DAY 24
BCNU	DAY 14 only	80	$^5/_5$	(-8.4)	NT	NT	NT	-	-	-
		40	$^0/_5$	-4.2	NT	NT	NT	-	-	-
		20	$^0/_5$	-1.0	NT	NT	NT	-	-	-
		10	$^0/_5$	-0.6	1.62 ± 0.71	0.44 ± 0.13	NM	98	11	≤2
		5	$^0/_5$	+0.3	1.81 ± 9.77	0.50 ± 0.18	NM	110	13	≤2
		2.5	$^0/_5$	+0.8	1.26 ± 0.46	0.72 ± 0.34	NM	76	18	≤2
		1.25	$^0/_5$	+0.5	1.32 ± 0.48	0.76 ± 0.33	NM	80	19	≤2
		0.63	$^0/_5$	+1.2	1.36 ± 0.64	1.39 ± 0.42	2.21 ± 2.09	82	35	39
		0.32	$^0/_5$	+1.8	1.59 ± 0.74	2.78 ± 1.17	4.98 ± 1.12	96	70	88
		0.16	$^0/_5$	+1.6	1.57 ± 0.65	3.59 ± 0.72	6.37 ± 1.09	95	91	113
		Control	$^0/_{17}$	+1.5	1.65 ± 0.59	3.95 ± 0.67	5.63 ± 1.05	100	100	100
	LD_{10} = 44 mg kg^{-1}				LD_{50} = 57 mg kg^{-1}			ID_{90} = 0.94 mg kg^{-1}		

Appendix 7.5c Activity of BCNU against the PC6 plasmacytoma

COMPOUND	Schedule	Dose $mgkg^{-1}$ day^{-1}	No. dead / Total	Mean body weight change (g)	Mean tumour volume (cm^3 ± s.d.) measured on days post implant			$\frac{T}{C}$ X 100%		
					DAY 14	DAY 19	DAY 24	DAY 14	DAY 19	DAY 24
HBDT	Days 14 - 19	320	5/5	-	-	-	-	-	-	-
		160	1/5	+1.4	1.91 ±0.78	3.60 ±0.54	6.29 ±1.17	116	91	112
		80	0/5	+1.0	1.53 ±0.81	3.48 ±0.74	6.28 ±1.01	93	88	112
		40	0/5	+1.3	1.69 ±0.93	4.00 ±1.16	6.97 ±1.53	102	101	124
		20	0/5	+1.4	1.53 ±0.46	3.66 ±0.50	5.63 ±0.58	93	93	100
		10	0/5	+1.3	1.74 ±0.39	4.21 ±0.64	6.16 ±1.25	105	107	109
		Control	0/17	+1.5	1.65 ±0.59	3.95 ±0.67	5.63 ±1.05	100	100	100
		LD_{10} = 147 $mgkg^{-1}day^{-1}$			LD_{50} = 208 $mgkg^{-1}day^{-1}$					
ABDT	Days 14 - 19	80	5/5	-	1.10 ±0.17	-	-	67	-	-
		40	1/5	+0.8	1.46 ±0.26	3.39 ±0.64	5.76 ±0.84	88	86	102
		20	0/5	+1.3	1.37 ±0.40	3.08 ±0.61	5.79 ±1.19	83	78	103
		10	0/5	+1.5	1.23 ±0.41	3.25 ±0.97	5.97 ±1.01	75	82	106
		5	0/5	+1.8	1.24 ±0.53	2.95 ±0.48	5.27 ±0.73	75	75	94
		Control	0/17	+1.5	1.65 ±0.59	3.95 ±0.67	5.63 ±1.05	100	100	100
		LD_{10} = 37 $mgkg^{-1}day^{-1}$			LD_{50} = 52 $mgkg^{-1}day^{-1}$					

Appendix 7.5d Activity of 2-substituted-4,6-bis(dimethylamino)-s-triazines against the PC6 plasmacytoma

X = 

COMPOUND	Schedule days of injection	Dose $mgkg^{-1}$ day^{-1}	No. dead / Total	Mean body weight change (g)	Mean tumour volume (cm^3 ± s.d.) measured on days post implant				$\frac{T}{C}$ x 100%			
					DAY 12	DAY 16	DAY 20	DAY 24	DAY 12	DAY 16	DAY 20	DAY 24
	1,3,5,7, 9,11,13, 15,17,19, 21	200	$^{1}/_{10}$	+1.5	1.04 ±0.18	1.93 ±0.29	3.45 ±0.54	5.04 ±0.58	91	107	98	100
		100	$^{0}/_{10}$	+0.5	1.00 ±0.27	1.65 ±0.33	3.30 ±0.53	4.96 ±0.81	88	91	94	95
		50	$^{0}/_{10}$	+0.1	0.92 ±0.21	1.68 ±0.34	3.20 ±0.57	4.78 ±0.68	81	93	91	95
		Control	$^{0}/_{32}$	+1.3	1.14 ±0.25	1.81 ±0.33	3.52 ±0.56	5.02 ±0.93	100	100	100	100
		$LD_{10} \sim 200$ mg $kg^{-1}day^{-1}$										
	1,3,5,7, 9,11,13, 15,17,19, 21	300	$^{0}/_{10}$	+0.2	0.98 ±0.20	1.80 ±0.33	3.34 ±0.56	5.12 ±0.85	86	99	95	102
		200	$^{0}/_{10}$	+0.6	0.98 ±0.21	1.83 ±0.17	3.41 ±0.48	4.91 ±0.99	86	101	97	98
		100	$^{0}/_{10}$	-0.7	0.87 ±0.19	1.52 ±0.37	2.99 ±0.62	4.71 ±1.12	76	84	85	94
		Control	$^{0}/_{32}$	+1.3	1.14 ±0.25	1.81 ±0.33	3.52 ±0.56	5.02 ±0.93	100	100	100	100
		Lethal doses not achieved										
	1,3,5,7, 9,11,13, 15,17,19, 21	300	$^{0}/_{10}$	-0.1	1.10 ±0.15	1.81 ±0.31	3.29 ±0.54	5.15 ±0.64	96	100	93	103
		200	$^{0}/_{10}$	+0.2	0.92 ±0.24	1.73 ±0.32	3.24 ±0.77	4.93 ±1.64	81	96	92	98
		100	$^{0}/_{10}$	+0.4	1.00 ±0.19	1.67 ±0.35	3.14 ±0.59	4.43 ±1.94	88	92	89	88
		Control	$^{0}/_{32}$	+1.3	1.14 ±0.25	1.81 ±0.33	3.52 ±0.56	5.02 ±0.93	100	100	100	100
		Lethal doses not achieved										

Appendix 7.6 Activity of 2-Sugar substituted-4,6-bis(dimethylamino)-s-triazines against the M5 sarcoma

COMPOUND	Schedule	Dose $mgkg^{-1}$ day^{-1}	No. Dead / Total	Mean body weight change (g)	Mean tumour volume (cm^3 ± s.d.) measured on days post implant				$\frac{T}{C}$ X 100%			
					DAY 12	DAY 16	DAY 20	DAY 24	DAY 12	DAY 16	DAY 20	DAY 24
76a	1,3,5,7,9,11,13,15,17,19,21	200	0/10	0	0.37±0.12	0.97±0.21	1.99±0.36	3.05±0.62	90	90	91	86
		100	0/10	-0.1	0.37±0.11	1.01±0.17	2.11±0.27	3.49±0.50	90	94	96	98
		50	0/10	+1.2	0.33±0.08	1.08±0.27	2.10±0.39	3.55±0.77	80	100	96	100
		Control	0/27	+0.8	0.41±0.10	1.08±0.18	2.19±0.40	3.55±0.64	100	100	100	100
76b	1,3,5,7,9,11,13,15,17,19,21	200	0/10	+0.3	0.40±0.10	1.17±0.18	2.04±0.36	3.13±0.64	98	108	93	88
		100	0/10	+0.8	0.43±0.13	1.07±0.23	2.00±0.52	2.96±0.71	105	99	91	83
		50	0/10	+1.1	0.38±0.11	1.13±0.24	2.19±0.47	3.23±0.49	93	106	100	91
		Control	0/27	+0.8	0.41±0.10	1.08±0.18	2.19±0.40	3.55±0.64	100	100	100	100
76c	1,3,5,7,9,11,13,15,17,19,21	200	0/10	+0.7	0.34±0.08	0.97±0.22	1.80±0.41	3.06±0.63	83	90	82	86
		100	0/10	+1.3	0.38±0.11	0.97±0.17	1.89±0.39	3.28±0.32	93	90	86	92
		50	0/10	+1.4	0.46±0.10	1.06±0.14	1.87±0.25	3.24±0.36	112	98	85	91
		Control	0/27	+0.8	0.41±0.10	1.08±0.18	2.19±0.40	3.55±0.64	100	100	100	100
76e	1,3,5,7,9,11,13 15,17,19,21	200	0/10	+0.1	0.48±0.15	0.81±0.12	1.24±0.19	2.43±0.22	107	94	75	86
		100	0/10	+0.3	0.56±0.17	0.86±0.15	1.40±0.42	2.33±0.55	124	100	85	83
		50	0/10	+0.3	0.49±0.15	0.76±0.27	1.44±0.28	2.45±0.43	109	88	87	87
		Control	0/28	+1.2	0.45±0.13	0.86±0.15	1.65±0.33	2.81±0.41	100	100	100	100
76f	1,3,5,7,9,11,13,15,17,19,21	200	0/10	+0.4	0.55±0.13	0.84±0.18	1.38±0.31	2.77±0.48	122	98	84	99
		100	0/10	+0.4	0.45±0.11	0.78±0.06	1.47±0.24	2.58±0.46	100	91	89	92
		50	0/10	+0.7	0.45±0.11	0.89±0.14	1.46±0.25	2.47±0.21	100	103	88	88
		Control	0/28	+1.3	0.45±0.13	0.86±0.15	1.65±0.33	2.81±0.41	100	100	100	100

Appendix 7.7 Activity of 2-substituted-benzylidenehydrazino-4,6-bis(dimethylamino)-s-triazines against the M5 sarcoma

COMPOUND	Schedule	Dose $mgkg^{-1}$ day^{-1}	No. Dead / Total	Mean body weight change (g)	Mean tumour volume (cm^3 ± s.d.) measured on days post implant				$\frac{T}{C}$ x 100%			
					DAY 12	DAY 16	DAY 20	DAY 24	DAY 12	DAY 16	DAY 20	DAY 24
77a	1,3,5,7,9,11,13,15,17,19,21	200	0/10	+0.6	0.42±0.09	1.05±0.17	1.77±0.39	3.07±0.43	102	97	81	86
		100	0/10	+1.4	0.42±0.10	1.09±0.19	1.95±0.16	3.23±0.56	102	101	89	91
		50	0/10	+1.2	0.38±0.10	1.11±0.14	2.18±0.83	3.61±0.41	93	103	100	102
		Control	0/27	+0.8	0.41±0.10	1.08±0.18	2.19±0.40	3.55±0.64	100	100	100	100
77b	1,3,5,7,9,11,13,15,17,19,21	200	1/10	+0.3	0.35±0.10	0.97±0.16	1.71±0.34	3.34±0.52	85	90	78	94
		100	0/10	+0.9	0.42±0.11	1.17±0.13	1.82±0.55	3.30±0.56	102	108	83	93
		50	0/10	+1.1	0.42±0.09	1.06±0.15	2.13±0.37	3.30±0.77	102	98	97	93
		Control	0/27	+0.8	0.41±0.10	1.08±0.18	2.19±0.40	3.55±0.64	100	100	100	100
78b	1,3,5,7,9,11,13,15,17,19,21	200	0/10	+0.3	0.41±0.13	1.16±0.27	1.84±0.52	3.42±0.58	100	107	84	96
		100	0/10	+1.1	0.46±0.09	1.10±0.17	2.25±0.54	3.50±0.53	112	102	103	99
		50	0/10	+1.0	0.36±0.10	1.08±0.25	2.30±0.37	3.32±0.44	88	100	105	94
		Control	0/27	+0.8	0.41±0.10	1.08±0.18	2.19±0.40	3.55±0.64	100	100	100	100
77c	1,3,5,7,9,11,13,15,17,19,21	300	0/10	-0.6	0.79±0.19	1.33±0.28	2.47±0.27	3.55±0.82	69	73	70	71
		200	0/10	-0.7	0.83±0.21	1.30±0.43	3.09±1.21	4.21±1.45	73	72	88	84
		100	0/10	-0.5	0.85±0.25	1.46±0.33	2.98±0.62	4.02±1.19	75	81	85	80
		Control	0/32	+1.3	1.14±0.25	1.81±0.33	3.52±0.56	5.02±0.93	100	100	100	100
78c	1,3,5,7,9,11,13,15,17,19,21	300	1/10	-1.0	0.88±0.16	1.47±0.44	2.72±0.74	3.73±1.18	77	81	77	74
		200	0/10	-0.8	0.92±0.26	1.43±0.38	2.94±0.82	4.61±1.19	81	79	84	92
		100	0/10	-0.4	0.94±0.29	1.61±0.42	3.05±0.68	4.68±0.79	82	89	87	93
		Control	0/32	+1.3	1.14±0.25	1.81±0.33	3.52±0.56	5.02±0.93	100	100	100	100

Appendix 7.8 Activity of the s-triazolo-1,3,5-triazines against the M5 sarcoma

Compound No.	Schedule Days of injection	Dose $mgkg^{-1}day^{-1}$	No.mice evaluable	Mean body weight change (g)	Median survival time (days)	$\frac{T}{C}$ X 100%	I.S.T.
64	1 - 4	200	3	0	9.5	100	-
		100	3	+2.3	9.5	100	-
		50	3	+2.6	9.5	100	-
37	1 - 4	200	3	-2.7	7.5	78	-
		100	3	-1.3	10.5	110	10%
		50	3	+0.3	9.5	100	-
67	1 - 4	200	3	-0.3	10.5	110	10%
		100	3	+0.7	9.5	100	-
		50	3	+1.7	9.5	100	-
65	1 - 4	200	3	-	-	-	-
		100	3	+2.3	9.2	96	-
		50	3	+1.7	10.2	107	7%
72	1 - 4	200	3	-0.7	8.5	89	-
		100	3	+0.3	7.5	78	-
		50	3	+1.7	8.2	86	-
76a	1 - 4	200	3	+0.7	6.5	68	-
		100	3	+0.3	11.2	117	17%
		50	3	+1.0	11.5	121	21%
76d	1 - 4	200	3	+1.0	10.0	105	5%
		100	3	+2.2	8.5	89	-
		50	3	+2.3	10.0	105	5%
76e	1 - 4	200	3	+1.7	9.5	100	-
		100	3	+2.5	9.3	97	-
		50	3	+2.0	10.5	110	10%
76f	1 - 4	200	3	-0.3	9.5	100	-
		100	3	-1.0	9.5	100	-
		50	3	+2.3	9.5	100	-
Control		-	40	+2.3	9.5	100	-

Appendix 7.9 Activity of analogues of HMM against the P388 leukaemia

Compound No.	Schedule days of injection	Dose $mgkg^{-1}day^{-1}$	No.mice evaluable	Mean body weight change (g)	Median survival time (days)	$\frac{T}{C}$ X 100%	I.S.T.
76b	1 - 4	200 100 50	3 3 3	-0.1 0 +1.3	12.0 13.0 11.0	126 136 115	26% 36% 15%
76c	1 - 4	200 100 50	3 3 3	+1.2 -0.3 +0.3	8.0 10.0 10.0	84 105 105	- 5% 5%
77a	1 - 4	200 100 50	3 3 3	0 +1.3 +2.0	10.2 10.0 9.0	107 105 94	7% 5% -
77b	1 - 4	200 100 50	3 3 3	+1.0 +1.0 +1.3	10.5 9.5 10.5	110 100 110	10% - 10%
78b	1 - 4	200 100 50	3 3 3	+1.0 +1.3 +0.7	8.5 9.2 10.5	89 96 110	- - 10%
77c	1 - 4	200 100 50	3 3 3	+0.7 +1.3 +3.7	11.0 11.5 10.3	115 121 106	15% 21% 6%
78c	1 - 4	200 100 50	3 3 3	+2.7 +3.7 +3.3	9.5 9.5 10.5	100 100 110	- - 10%
Control		-	40	+2.3	9.5	100	

Appendix 7.9 Activity of analogues of HMM against the P388 leukaemia (cont.)

Compound No.	Schedule days of injection	Dose $mgkg^{-1}day^{-1}$	No.mice evaluable	Mean body weight change (g)	Median survival time (days)	$\frac{T}{C}$ X 100%	I.S.T.
76a	1 - 9	200	6	-2. 0	10.0	85	-
		100	6	-2.7	9.3	79	-
		50	6	-2.3	8.8	75	-
		25	6	-2.1	9.3	79	-
76b	1 - 9	300	6	-1.1	11.9	101	1%
		200	6	-0.8	12.2	103	3%
		100	6	-0.2	12.0	102	2%
		50	6	-1.9	11.9	101	1%
77c	1 - 9	300	6	-3.0	10.0	84	-
		200	6	-2.8	9.9	84	-
		100	6	-2.5	9.7	82	-
		50	6	-2.2	9.2	80	-
Control	-	-	40	+0.7	11.8	-	
76a	1 - 5	200	6	+1.5	11.3	96	-
		100	6	+2.5	8.3	70	-
		50	6	+2.0	10.3	87	-
		25	6	+2.2	9.3	79	-
76b	1 - 5	400	6	+0.4	11.3	96	-
		200	6	+1.4	11.3	96	-
		100	6	+0.7	12.3	104	4%
		50	6	+0.7	12.3	104	4%
77c	1 - 5	400	6	+2.2	11.7	99	-
		300	6	+2.2	11.0	93	-
		200	6	+2.0	11.7	99	-
		100	6	+2.2	10.7	91	-
Control	-	-	40	+0.7	11.8		

Appendix 7.9 Activity of analogues of HMM against the P388 leukaemia (cont.)

COMPOUND	Dose $mgkg^{-1}$	Assay temperature ^{o}C	Formaldehyde equivalents ($nmoles\ ml^{-1}$) at time points post injection						
			10 min	30 min	1h	$1\frac{1}{2}$h	2h	4h	8h
HMM	100	0^{o}	0	3 ± 3	3 ± 3	0	0	0	0
		60^{o}	72 ± 27	99 ± 15	111 ± 3	87 ± 3	51 ± 24	21 ± 9	12 ± 6
PMM	93	0^{o}	6 ± 6	12 ± 9	12 ± 15	3 ± 6	3 ± 6	0	0
		60^{o}	126 ± 30	243 ± 27	192 ± 18	108 ± 30	48 ± 39	0	0
TMM	86	0^{o}	0	3 ± 6	0	0	0	-	-
		60^{o}	81 ± 27	101 ± 15	60 ± 24	33 ± 27	3 ± 3	0	0
TriMM	80	0^{o}	0	0	0	6 ± 6	3 ± 6	0	-
		60^{o}	24 ± 15	51 ± 18	36 ± 15	6 ± 9	9 ± 6	0	-
CBDT	96	0^{o}	3 ± 3	3 ± 3	0	0	3 + 3	-	-
		60^{o}	0	15 ± 27	15 ± 27	6 ± 9	0	-	-
HBDT	94	0^{o}	0	6 ± 3	3 ± 3	15 ± 3	3 ± 3	0	0
		60^{o}	0	33 ± 9	33 ± 3	33 ± 18	0	0	0

Appendix 8.1 Plasma levels of formaldehyde precursors generated by the analogues of HMM

COMPOUND	Schedule	Dose $mgkg^{-1}$ inj^{-1}	Toxic Deaths / Total	Median Survival Day	$\frac{T}{C}$ X 100%	% I.S.T.
FORMALDEHYDE	Once daily 1 - 9	40	$^{0}/_{10}$	13.6	135	35
	Twice daily 1 - 9	30	$^{3}/_{10}$	12.0	119	19
		25	$^{1}/_{10}$	13.3	132	32
	Three times daily 1 - 9	25	$^{4}/_{10}$	9.5	94	-
		20	$^{0}/_{10}$	13.6	135	35
		15	$^{0}/_{10}$	13.3	132	32
CONTROL	-	-	$^{0}/_{66}$	10.1	100	-
FORMALDEHYDE	Once daily 1 - 9	40	$^{0}/_{10}$	13.2	131	31
CONTROL	- -	-	$^{0}/_{94}$	10.1	100	-

Appendix 8.2a Activity of formaldehyde against the P388 leukaemia

COMPOUND	Schedule	Dose $mgkg^{-1}$ inj^{-1}	Toxic Deaths / Total	Median Survival Day	$\frac{T}{C}$ X 100%	% I.S.T.
FORMALDEHYDE	Once daily 1 - 8	40	$^{0}/_{10}$	9.0	118	18
CONTROL	-		$^{0}/_{30}$	7.6	100	-

Appendix 8.2b Activity of formaldehyde against the L1210 leukaemia

COMPOUND	Schedule days of injection	Dose $mgkg^{-1}$ inj^{-1}	Mean body weight change (g)	Toxic Deaths / Total	Median survival day	$\frac{T}{C}$X100%	% IST
FORMALDEHYDE	1 - 9	40	-3.2	$^{0}/_{10}$	21.2	100	-
CONTROL	-	-	+0.4	$^{0}/_{28}$	21.3	100	-

Appendix 8.2c Activity of formaldehyde against the 816 melanoma

TUMOUR FORM	Schedule	Dose $mgkg^{-1}$ day^{-1}	Mean body weight change(g)	Toxic deaths / Total	Median survival day	$\frac{T}{C}$ X 100%	% I.S.T.	Cures
Ascites i.p.	Twice daily 1 - 5	30	+0.1	$^{3}/_{16}$	27.0	148	48	$^{3}/_{16}$
		Control	+2.0	$^{0}/_{11}$	18.3	100	-	
	Twice daily 1 - 5	30	-1.4	$^{0}/_{8}$	26.0	141	41	$^{3}/_{8}$
		Control	+2.7	$^{0}/_{10}$	18.5	100	-	
	Once daily 1 - 5	35	-0.7	$^{0}/_{10}$	19.0	98	-	
		Control	+1.0	$^{0}/_{16}$	19.3	100	-	
	Once daily 1 - 9	40	-	$^{4}/_{10}$	18.3	151	51	
		30	-	$^{0}/_{10}$	33.5	178	78	
		Control	-	$^{0}/_{10}$	18.8	100	-	

Appendix 8.2d Activity of formaldehyde against the ascitic (i.p.) Sarcoma 180

TUMOUR FORM	Schedule	Dose $mgkg^{-1}$ day^{-1}	No. Dead / Total	Mean body weight change (g)	Mean tumour volume (cm^3) measured on days post implant				$\frac{T}{C}$ X 100%				Median survival Day	$\frac{T}{C}$ X 100%	% I.S.T.
					DAY 7	DAY 10	DAY 14	DAY 19	DAY 7	DAY 10	DAY 14	DAY 19			
Solid i.m.	Twice daily 1 - 9	40	$^{9}/_{10}$	-0.8	0.35	–	–	–	59	–	–	–	8.2	37	
		30	$^{0}/_{10}$	-0.8	0.64	1.56	2.27	3.73	108	110	97	87	25.0	114	14
		25	$^{0}/_{10}$	-0.4	0.59	1.39	2.26	3.88	100	97	97	90	24.0	109	9
		Control	$^{0}/_{40}$	+1.2	0.59	1.43	2.33	4.30	100	100	100	100	22.0	100	

Appendix 8.2e Activity of formaldehyde against the solid (i.m.) Sarcoma 180

COMPOUND	Schedule Days of injection	Dose $mgkg^{-1}$ day^{-1}	Mean body weight change (g)	No. Dead / Total	Mean tumour volume (cm^3 ± s.d.) measured on days post implant				$\frac{T}{C}$ X 100%			
					DAY 12	DAY 16	DAY 20	DAY 24	DAY 12	DAY 16	DAY 20	DAY 24
FORMALDEHYDE	Twice daily 1 - 11	22.5	-1.4	2/10	0.49 ±0.24	1.09 ±0.30	1.82 ±0.42	2.79 ±0.64	84	95	86	83
		15.0	-0.6	0/10	0.50 ±0.31	1.04 ±0.25	1.80 ±0.43	2.92 ±0.69	86	90	85	87
		7.5	0	0/10	0.58 ±0.17	1.04 ±0.30	2.17 ±0.59	3.27 ±0.67	100	90	103	97
		Control	+1.0	0/30	0.58 ±0.39	1.15 ±0.59	2.11 ±0.67	3.36 ±0.69	100	100	100	100
	Twice daily 1 - 17	25	-1.5	5/10	0.26 ±0.06	0.42 ±0.14	1.20 ±0.26	2.00 ±0.40	72	51	72	80
		20	-0.7	0/10	0.27 ±0.06	0.57 ±0.10	1.43 ±0.29	2.21 ±0.46	75	69	86	89
		15	+0.1	0/10	0.31 ±0.08	0.70 ±0.18	1.38 ±0.32	2.12 ±0.34	86	84	83	85
		Control	+0.5	0/27	0.36 ±0.09	0.83 ±0.17	1.66 ±0.35	2.49 ±0.48	100	100	100	100
	Eight times daily 1,5,9	30	-3.0	4/10	0.51 ±0.14	1.28 ±0.37	1.74 ±0.39	2.86 ±0.70	96	112	99	105
		20	-1.8	4/10	0.32 ±0.09	0.82 ±0.31	1.39 ±0.38	2.45 ±0.65	60	72	79	90
		10	-1.8	0/10	0.46 ±0.11	0.87 ±0.25	1.43 ±0.30	2.32 ±0.42	87	76	82	85
		Control	+0.7	0/35	0.53 ±0.14	1.14 ±0.26	1.75 ±0.40	2.73 ±0.56	100	100	100	100

Appendix 8.2f Activity of formaldehyde against the M5 sarcoma

COMPOUND Route and Site		Schedule days of injection	Dose $mgkg^{-1}$ day^{-1}	Mean body weight change (g)	Mean tumour volume (cm^3 ± s.d.) measured on days post implant					$\frac{T}{C}$ X 100%				
					DAY 12	DAY 16	DAY 20	DAY 25	DAY 31	DAY 12	DAY 16	DAY 20	DAY 25	DAY 31
FORMALDEHYDE	i.m. left leg	1 - 5 13 - 17	40	+0.6	0.27 ±0.08	0.57 ±0.18	0.57 ±0.21	0.83 ±0.37	1.36 ±0.52	51	50	33	30	29
			30	+0.2	0.23 ±0.09	0.45 ±0.17	0.52 ±0.23	0.81 ±0.34	1.36 ±0.66	43	39	30	30	29
	i.m. right leg	1 - 5 13 - 17	40	+0.4	0.48 ±0.11	0.98 ±0.24	1.49 ±0.26	2.70 ±0.35	4.39 ±0.85	91	86	85	99	92
			30	0	0.47 ±0.12	0.89 ±0.28	1.45 ±0.52	2.50 ±0.73	4.43 ±1.16	89	78	83	92	93
HEXAMETHYLMELAMINE	i.m. left leg	1 - 5 13 - 17	80	-0.2	0.27 ±0.09	0.46 ±0.10	0.76 ±0.18	1.23 ±0.35	2.05 ±0.40	51	40	43	45	43
			60	-0.5	0.25 ±0.07	0.42 ±0.17	0.74 ±0.26	1.41 ±0.37	2.42 ±0.52	47	37	42	52	51
	i.m. right leg	1 - 5 13 - 17	80	-1.0	0.24 ±0.08	0.43 ±0.14	0.82 ±0.25	1.38 ±0.31	1.97 ±0.29	45	38	47	51	41
			60	-0.3	0.24 ±0.03	0.49 ±0.14	0.89 ±0.31	1.62 ±0.39	2.61 ±0.38	45	43	51	59	55
Control				+0.6	0.53 ±0.14	1.14 ±0.26	1.75 ±0.40	2.73 ±0.56	4.76 ±0.79	100	100	100	100	100

Appendix 8.3/8.4 Activities of formaldehyde and HMM injected at different sites against the M5 sarcoma

COMPOUND	Route	Schedule Day of injection	Dose $mgkg^{-1}$ day^{-1}	Mean body weight change (g)	Mean tumour volume (cm^3 ± s.d.) measured on days post implant			$\frac{T}{C}$ X 100%		
					DAY 14	DAY 19	DAY 24	DAY 14	DAY 19	DAY 24
HEXAMETHYLMELAMINE	i.p.	14	90	+0.1	1.17 ±0.18	0.96 ±0.26	0.24 ±0.14	87	32	5
	p.o.	14	90	+1.0	1.24 ±0.20	1.40 ±0.42	1.93 ±0.83	92	47	37
	s.c. (flank)	14	90	+1.1	1.31 ±0.26	1.78 ±0.21	2.30 ±1.16	97	60	44
	i.m. left leg	14	90	+0.1	1.38 ±0.22	1.53 ±0.43	1.65 ±1.65	102	51	31
	i.m. right leg	14	90	+0.1	1.20 ±0.16	1.34 ±0.23	1.53 ±0.55	89	45	29
	Control			+1.6	1.35 ±0.40	2.98 ±0.36	5.25 ±0.92	100	100	100

Appendix 8.5 Activity of HMM (administered by a variety of routes) against the PC6 plasmacytoma

SCHEDULE Days of injection	DOSE mg kg^{-1} day^{-1}	Mean body weight change (g)	MEAN TUMOUR VOLUME (cm^3 ± s.d.) measured on days post implant						$\frac{T}{C}$ x 100%					
			DAY 12	DAY 16	DAY 20	DAY 25	DAY 28	DAY 31	DAY 12	DAY 16	DAY 20	DAY 25	DAY 28	DAY 31
A 1 - 15	133	+ 2.2	NM	NM	NM	0.73 ±0.12	1.25 ±0.30	2.19 ±0.50	-	-	⩽5	18	24	36
B 1 - 10	200	+ 1.6	NM	NM	0.44 ±0.11	1.03 ±0.10	1.91 ±0.16	2.78 ±0.75	-	⩽10	17	25	37	46
C 1 - 5: 10 - 15	200	+ 2.0	NM	NM	0.22 ±0.09	0.97 ±0.25	1.74 ±0.43	2.26 ±0.61	-	⩽10	9	24	33	37
D 1,3,5,7, 9,11,13, 15,17	220	+ 1.4	NM	NM	0.16 ±0.04	0.80 ±0.15	1.13 ±0.18	2.07 ±0.24	-	⩽10	6	20	22	34
E 1,5,9,13, 17	400	+ 2.9	NM	0.18 ±0.06	0.24 ±0.10	1.01 ±0.35	1.57 ±0.45	2.34 ±0.45	⩽24	14	9	25	30	38
F 1 - 5	400	+ 1.8	NM	NM	0.77 ±0.23	2.16 ±0.37	3.06 ±0.46	4.38 ±0.63	-	⩽10	30	53	59	72
G 1,9,17	667	+ 2.3	NM	0.36 ±0.06	0.44 ±0.06	1.62 ±0.32	2.27 ±0.19	2.99 ±0.27	⩽24	28	17	40	43	49
H 1,9	1000	+ 1.1	NM	0.27 ±.10	0.96 ±0.12	1.96 ±0.31	2.80 ±0.44	3.58 ±0.41	⩽24	21	38	48	54	59
I 1,17	1000	+ 1.0	0.22 ±0.07	0.65 ±0.09	0.50 ±0.11	1.81 ±0.39	2.49 ±0.41	3.61 ±0.42	42	50	20	44	48	59
J 1	1000	+ 1.1	0.29 ±0.01	0.67 ±0.13	1.68 ±0.28	2.66 ±0.45	3.58 ±0.87	4.62 ±0.82	55	52	66	65	69	76
Control	-	+ 3.0	0.53 ±0.10	1.30 ±0.30	2.56 ±0.33	4.07 ±0.46	5.22 ±0.34	6.08 ±0.42	100	100	100	100	100	100

NM = Non measurable

Appendix 9.1 Schedule dependency of NMF against the M5 sarcoma

SCHEDULE Days of injection	DOSE mg kg^{-1} day^{-1}	Mean Body Weight change (g)	MEAN TUMOUR VOLUME (cm^3 ± s.d.) measured on days post implant								
			DAY12	DAY 16	DAY 20	DAY 24	DAY 29	DAY 36	DAY 45	DAY 51	DAY 55
1 - 5 11 - 15 21 - 25 31 - 35 41 - 45 51 - 55	200	+ 4.9	NM	NM	0.29 ±0.15	NM	0.50 ±0.14	0.44 ±0.16	1.01 ±0.38	2.79 ±1.03	1.43 ±0.42
Control			0.34 ±0.08	1.12 ±0.26	2.26 ±0.55	3.28 ±0.74					

NM = Non measurable

Appendix 9.2 NMF against the M5 sarcoma on a 5 day on/5 day off schedule

COMPOUND	SCHEDULE Days of injection	DOSE mg kg^{-1} day^{-1}	No. Dead / Total	Mean Body Weight Change (g)	MEAN TUMOUR VOLUME (cm^3 ± s.d.) measured on days post implant				$\frac{T}{C}$ X 100%			
					DAY 12	DAY 16	DAY 20	DAY 24	DAY 12	DAY 16	DAY 20	DAY 24
N-METHYLFORMAMIDE	1 - 17	400	7/8	- 5.9	NM	NM	NM	NM				⩽5
		300	4/8	- 2.3	NM	NM	NM	NM				⩽5
		200	0/8	- 0.3	NM	NM	NM	NM				⩽5
		100	0/8	+ 0.7	NM	NM	NM	0.27 ±0.11			⩽7	10
		50	0/8	+ 1.8	NM	NM	0.27 ±0.12	0.88 ±0.16		⩽13	15	32
		Control	0/18	+ 2.6	0.33 ±0.05	0.94 ±0.15	1.86 ±0.26	2.72 ±0.48	100	100	100	100
	1 - 17	25	0/10	+ 2.0	NM	0.43 ±0.08	1.16 ±0.30	2.09 ±0.55	⩽48	49	65	71
		12.5	0/10	+ 1.6		0.62 ±0.12	1.53 ±0.35	2.36 ±0.61		71	85	80
		6.25	0/10	+ 1.7		0.79 ±0.13	1.69 ±0.35	2.66 ±0.43		91	94	90
		Control	0/18	+ 2.7	0.26 ±0.12	0.87 ±0.20	1.79 ±0.32	2.95 ±0.56	100	100	100	100
		LD_{10} = 220 mgkg^{-1}day^{-1} LD_{50} = 300 mgkg^{-1}day^{-1} ID_{90} = 100 mgkg^{-1}day^{-1}										
FORMAMIDE	1 - 17	400	10/10	-	-	-	-	-	-	-	-	-
		200	1/10	+ 0.5	0.13 ±0.03	0.18 ±0.05	0.36 ±0.13	0.76 ±0.27	38	25	28	37
		100	0/10	+ 0.9	0.16 ±0.04	0.28 ±0.08	0.57 ±0.16	1.12 ±0.19	47	38	44	54
		Control	0/27		0.34 ±0.09	0.73 ±0.19	1.29 ±0.34	2.06 ±0.62	100	100	100	100
		LD_{10} = 200 mgkg^{-1}day^{-1} LD_{50} = 270 mgkg^{-1}day^{-1}										

Appendix 9.3 Activity of N-alkylformamides against the M5 sarcoma

COMPOUND	SCHEDULE Days of Injection	DOSE mg kg^{-1} day^{-1}	No. dead / Total	Mean body weight change (g)	MEAN TUMOUR VOLUME (cm^3 ± s.d.) measured on days post implant				$\frac{T}{C}$ X 100%			
					DAY 12	DAY 16	DAY 20	DAY 24	DAY 12	DAY 16	DAY 20	DAY 24
N,N-DIMETHYLFORMAMIDE	1 - 17	1500	$^{10}/_{10}$	-	-	-	-	-	-	-	-	-
		1200	$^{3}/_{10}$	+ 0.7	NM	0.15 ±0.04	0.23 ±0.12	0.68 ±0.25	⩽37	21	18	33
		Control	$^{0}/_{27}$		0.34 ±0.09	0.73 ±0.19	1.29 ±0.34	2.06 ±0.62	100	100	100	100
	1 - 17	1000	$^{0}/_{10}$	+ 0.2	0.13 ±0.02	0.17 ±0.06	0.32 ±0.09	0.99 ±0.17	36	20	19	40
		800	$^{0}/_{10}$	+ 0.1	0.15 ±0.05	0.19 ±0.08	0.46 ±0.33	1.38 ±0.40	42	23	28	55
		600	$^{0}/_{10}$	+ 0.6	0.16 ±0.04	0.24 ±0.06	0.65 ±0.33	1.54 ±0.40	44	29	39	62
		Control	$^{0}/_{27}$	+ 0.5	0.36 ±0.09	0.83 ±0.17	1.66 ±0.35	2.49 ±0.48	100	100	100	100
		LD_{10} = 1130 mg $kg^{-1}day^{-1}$			LD_{50} = 1280 mg $kg^{-1}day^{-1}$							
N-ETHYLFORMAMIDE	1 - 17	600	$^{10}/_{10}$	-	-	-	-	-	-	-	-	-
		300	$^{0}/_{10}$		0.25 ±0.09	0.43 ±0.12	1.08 ±0.34	1.95 ±0.46	69	52	65	78
		Control	$^{0}/_{10}$		0.36 ±0.09	0.83 ±0.17	1.66 ±0.35	2.49 ±0.48	100	100	100	100
	1 - 17	200	$^{0}/_{10}$	+ 0.8	0.37 ±0.08	0.74 ±0.20	1.57 ±0.37	2.59 ±0.58	70	74	79	85
		100	$^{0}/_{10}$	+ 0.7	0.51 ±0.09	0.89 ±0.20	1.74 ±0.37	2.83 ±0.58	96	89	88	93
		Control	$^{0}/_{15}$	+ 0.5	0.53 ±0.16	1.00 ±0.24	1.98 ±0.34	3.05 ±0.61	100	100	100	100
		LD_{10} = 320 mg $kg^{-1}day^{-1}$			LD_{50} = 420 mg $kg^{-1}day^{-1}$							

Appendix 9.3 Activity of N-alkylformamides against the M5 sarcoma (cont.)

COMPOUND	SCHEDULE Days of Injection	DOSE mg kg^{-1} day^{-1}	NO. DEAD / Total	Mean Body Weight Change (g)	MEAN TUMOUR VOLUME (cm^3 ± s.d.) measured on days post implant				$\frac{T}{C}$ X 100%			
					DAY 12	DAY 16	DAY 20	DAY 24	DAY 12	DAY 16	DAY 20	DAY 24
N-HYDROXYMETHYL FORMAMIDE	1 - 17	2500	5/5	-	-	-	-	-	-	-	-	-
	1 - 17	2000	3/5	- 5.1	NM	NM	NM	0.54 ±0.10			⩽5	17
		Control	0/19	+ 1.9	0.45 ±0.11	1.05 ±0.23	2.30 ±0.45	3.20 ±0.54	100	100	100	100
		1500	0/6	0	0.17 ±0.04	0.20 ±0.06	0.40 ±0.05	0.77 ±0.09	50	27	31	37
		1000	0/10	+ 0.4	0.18 ±0.04	0.26 ±0.05	0.51 ±0.14	0.96 ±0.26	53	36	40	47
		600	0/10	+ 0.5	0.22 ±0.07	0.41 ±0.12	0.84 ±0.17	1.39 ±0.29	65	56	65	67
		Control	0/27	+ 1.6	0.34 ±0.09	0.73 ±0.19	1.29 ±0.34	2.06 ±0.61	100	100	100	100
		LD_{10} = 1580 mg $kg^{-1}day^{-1}$			LD_{50} = 1930 mg $kg^{-1}day^{-1}$							
N-HYDROXYMETHYL-N-METHYLFORMAMIDE	1 - 17	1200	5/5	-	-	-	-	-	-	-	-	-
		800	5/5	-	NM	-	-	-	-	-	-	-
		400	1/5	+ 0.4	0.16 ±0.04	0.30 ±0.13	0.89 ±0.12	2.43 ±0.39	36	29	39	76
	1 - 17	200	0/5	+ 1.9	0.21 ±0.06	0.45 ±0.08	1.32 ±0.27	2.55 ±0.56	47	43	57	80
		100	0/5	+ 1.4	0.36 ±0.07	0.73 ±0.09	1.69 ±0.35	2.70 ±0.63	80	70	73	84
		Control	0/19	+ 1.9	0.45 ±0.11	1.05 ±0.23	2.30 ±0.45	3.20 ±0.54	100	100	100	100
		LD_{10} = 370 mg $kg^{-1}day^{-1}$			LD_{50} = 520 mg $kg^{-1}day^{-1}$							

Appendix 9.3 Activity of N-hydroxymethylformamides against the M5 sarcoma.

COMPOUND	SCHEDULE Days of Injection	DOSE mg kg^{-1} day^{-1}	NO. DEAD / Total	Mean Body Weight Change (g)	MEAN TUMOUR VOLUME (cm^3 ± s.d.) measured on days post implant				$\frac{T}{C}$ X 100%			
					DAY 6	DAY 10	DAY 13	DAY 16	DAY 6	DAY 10	DAY 13	DAY 16
N-METHYL FORMAMIDE	1 - 9	600	$^{10}/_{10}$	-	-	-	-	-	-	-	-	-
		450	$^{10}/_{10}$	-	-	-	-	-	-	-	-	-
		300	$^{0}/_{10}$	-3.2	NM	0.22 ±0.04	0.45 ±0.14	0.89 ±0.32	⩽36	24	35	48
		Control	$^{0}/_{20}$	0	0.35 ±0.08	0.91 ±0.21	1.28 ±0.30	1.86 ±0.39	100	100	100	100
FORMAMIDE	1 - 9	450	$^{7}/_{10}$	-2.1	0.14 ±0.04	0.32 ±0.08	0.64 ±0.23	1.14 ±0.35	37	37	37	52
		300	$^{0}/_{10}$	-1.1	0.23 ±0.04	0.45 ±0.07	0.78 ±0.15	1.15 ±0.27	61	52	45	53
		Control	$^{0}/_{31}$	+1.0	0.38 ±0.09	0.87 ±0.17	1.72 ±0.26	2.18 ±0.37	100	100	100	100
NN-DIMETHYL FORMAMIDE	1 - 9	1500	$^{10}/_{10}$	-	-	-	-	-	-	-	-	-
		1000	$^{0}/_{10}$	+2.2	0.26 ±0.05	0.76 ±0.11	1.52 ±0.23	2.26 ±0.3	68	87	88	104
		Control	$^{0}/_{31}$	+1.0	0.38 ±0.09	0.87 ±0.17	1.72 ±0.26	2.18 ±0.37	100	100	100	100
N-ETHYL FORMAMIDE	1 - 9	600	$^{5}/_{10}$	-3.9	-	-	-	-	-	-	-	-
		400	$^{0}/_{10}$	-0.7	0.19 ±0.05	0.59 ±0.10	1.10 ±0.18	1.68 ±0.24	50	68	64	77
		300	$^{0}/_{10}$	+0.7	0.29 ±0.05	0.70 ±0.04	1.38 ±0.14	1.94 ±0.24	76	80	80	89
		Control	$^{0}/_{31}$	+1.0	0.38 ±0.09	0.87 ±0.17	1.72 ±0.26	2.18 ±0.37	100	100	100	100

Appendix 9.4 The activity of N-alkylformamides against the Sarcoma 180.

COMPOUND	SCHEDULE Days of injection	DOSE mg kg^{-1} day^{-1}	NO. DEAD / Total	Mean Body weight change (g)	MEAN TUMOUR VOLUME (cm^3 ± s.d.) measured on days post implant				$\frac{T}{C}$ X 100%			
					DAY 12	DAY 16	DAY 20	DAY 24	DAY 12	DAY 16	DAY 20	DAY 24
NN-DIMETHYLACETAMIDE	1 - 17	1600	$^3/_5$	+ 0.8	0.20	0.51 ±0.14	1.10	2.16 ±0.06	59	59	63	77
		800	$^0/_5$	+ 2.1	0.29 ±0.10	0.54 ±0.07	1.11 ±0.13	2.20 ±0.43	65	62	63	78
		400	$^0/_5$	+ 2.3	0.32 ±0.04	0.60 ±0.03	1.41 ±0.10	2.45 ±0.034	94	69	80	87
		200	$^0/_5$	+ 2.6	0.31 ±0.11	0.76 ±0.12	1.58 ±0.17	2.77 ±0.26	91	87	90	99
		100	$^0/_5$	+ 2.1	0.25 ±0.08	0.61 ±0.21	1.24 ±0.18	2.30 ±0.42	74	70	70	82
		Control	$^0/_{18}$	+ 2.4	0.34 ±0.07	0.87 ±0.16	1.76 ±0.30	2.81 ±0.44	100	100	100	100
		LD_{10} = 900 mg kg^{-1}day^{-1} LD_{50} = 1420 mg kg^{-1}day^{-1}										
NNN'N'-TETRA-METHYLGUANIDINE	1 - 17	800	$^5/_5$	-	-	-	-	-	-	-	-	-
		400	$^5/_5$	-	-	-	-	-	-	-	-	-
		200	$^5/_5$	-	-	-	-	-	-	-	-	-
		100	$^0/_5$	+ 1.8	0.32 ±0.05	1.53 ±0.37	2.74 ±0.42	4.17 ±0.41	94	138	121	127
		50	$^0/_5$	+ 3.4	0.30 ±0.96	1.40 ±0.26	2.55 ±0.41	3.81 ±0.19	88	125	113	116
		Control	$^0/_{17}$	+ 1.9	0.34 ±0.08	1.12 ±0.26	2.26 ±0.55	3.28 ±0.74	100	100	100	100
		LD_{10} = 107 mg kg^{-1}day^{-1} LD_{50} = 142 mg kg^{-1}day^{-1}										

Appendix 9.5 Activity of derivatives of acetamide and urea against the M5 sarcoma

COMPOUND	SCHEDULE Days of injection	DOSE mg kg^{-1} day^{-1}	NO. DEAD / Total	Mean Body weight change (g)	MEAN TUMOUR VOLUME (cm^3 ± s.d.) measured on days post implant				$\frac{T}{C}$ X 100%			
					DAY 12	DAY 16	DAY 20	DAY 24	DAY 12	DAY 16	DAY 20	DAY 24
NN'-DIMETHYLUREA	1 - 17	3200	5/5	-	-	-	-	-	-	-	-	-
		1600	0/5	+ 0.4	0.23 ±0.10	0.57 ±0.15	1.60 ±0.26	2.11 ±0.27	51	54	70	66
		Control	0/19	+ 1.9	0.45 ±0.11	1.05 ±0.23	2.30 ±0.45	3.20 ±0.54	100	100	100	100
	1 - 17	800	0/8	+ 1.1	0.21 ±0.05	0.59 ±0.16	1.21 ±0.17	2.75 ±0.48	70	69	74	93
		400	0/8	+ 1.8	0.22 ±0.05	0.75 ±0.17	1.22 ±0.25	2.64 ±0.52	73	88	75	89
		200	0/8	+ 1.3	0.33 ±0.06	0.94 ±0.20	1.60 ±0.45	3.23 ±0.82	110	110	98	109
		100	0/8	+ 1.8	0.30 ±0.05	0.85 ±0.11	1.36 ±0.13	2.56 ±0.41	100	100	83	86
		50	0/8	+ 1.7	0.28 ±0.07	0.91 ±0.18	1.51 ±0.33	3.01 ±0.46	93	107	93	102
		Control	0/11	+ 2.3	0.30 ±0.05	0.85 ±0.24	1.63 ±0.38	2.96 ±0.56	100	100	100	100
		LD_{10} = 1730 mg $kg^{-1}day^{-1}$					LD_{50} = 2260 mg $kg^{-1}day^{-1}$					
NNN'N'-TETRA-METHYLUREA	1 - 17	800	6/8	- 3.8	0.16 ±0.03	0.17 ±0.01	0.42	1.20 ±0.35	53	20	26	41
		400	0/8	+ 0.6	0.23 ±0.05	0.46 ±0.09	1.03 ±0.21	1.98 ±0.51	77	54	63	67
		200	0/8	+ 1.5	0.25 ±0.04	0.74 ±0.18	1.51 ±0.34	2.77 ±0.58	83	87	93	94
		100	0/8	+ 2.1	0.28 ±0.05	0.77 ±0.13	1.39 ±0.33	2.75 ±0.40	93	91	85	93
		50	0/8	+ 2.6	0.27 ±0.06	0.68 ±0.19	1.46 ±0.30	2.83 ±0.62	90	80	90	96
		Control	0/11	+ 2.3	0.30 ±0.05	0.85 ±0.24	1.63 ±0.38	2.96 ±0.56	100	100	100	100
		LD_{10} = 440 mg $kg^{-1}day^{-1}$					LD_{50} = 640 mg $kg^{-1}day^{-1}$					

Appendix 9.5 Activity of derivatives of acetamide and urea against the M5 sarcoma (cont.)

COMPOUND	SCHEDULE Days of injection	DOSE mg kg^{-1} day^{-1}	NO. DEAD / Total	Mean Body weight change (g)	MEAN TUMOUR VOLUME (cm^3 ± s.d.) measured on days post implant				$\frac{T}{C}$ x 100%			
					DAY 12	DAY 16	DAY 20	DAY 24	DAY 12	DAY 16	DAY 20	DAY 24
NN-DIMETHYL-THIOFORMAMIDE	1 - 17	400	$^5/_5$	-	-	-	-	-	-	-	-	-
		200	$^1/_5$	+ 1.1	0.25 ±0.06	0.56 ±0.09	1.88 ±0.19	3.09 ±0.51	56	53	82	97
		100	$^0/_5$	+ 2.5	0.31 ±0.09	0.63 ±0.19	1.53 ±0.53	2.47 ±0.33	69	60	67	77
		50	$^0/_5$	+ 2.8	0.35 ±0.10	0.98 ±0.12	2.97 ±0.20	2.77 ±0.32	78	93	90	87
		Control	$^0/_{19}$	+ 1.9	0.45 ±0.11	1.05 ±0.23	2.30 ±0.45	3.20 ±0.54	100	100	100	100
		LD_{10}= 185 mg kg^{-1}day^{-1}					LD_{50} = 260 mg kg^{-1}day^{-1}					
NN'-DIMETHYL-THIOUREA	1 - 17	800	$^3/_5$	+ 1.4	NM	0.78 ±0.16	2.42 ±0.32	3.50 ±0.49	28	58	91	80
		400	$^0/_5$	+ 2.2	0.25 ±0.05	1.05 ±0.12	2.54 ±0.30	3.96 ±0.55	⩽57	78	96	91
		200	$^0/_5$	+ 1.2	0.36 ±0.11	1.47 ±0.24	2.98 ±0.44	4.46 ±0.42	82	110	112	102
		100	$^0/_5$	+ 1.5	0.44 ±0.07	1.45 ±0.21	2.41 ±0.47	3.98 ±0.82	100	108	91	91
		Control	$^0/_{16}$	+ 2.3	0.44 ±0.07	1.34 ±0.23	2.65 ±0.33	4.36 ±0.51	100	100	100	100
		LD_{10} = 450 mg kg^{-1}day^{-1}					LD_{50} = 720 mg kg^{-1}day^{-1}					
NNN'N'-TETRA-METHYLTHIOUREA	1 - 17	800	$^5/_5$	-	-	-	-	-	-	-	-	-
		400	$^0/_5$	- 3.0	0.23 ±0.04	0.61 ±0.16	1.89 ±0.32	2.53 ±0.66	51	58	82	79
		200	$^0/_5$	+ 1.3	0.41 ±0.06	0.91 ±0.20	2.32 ±0.18	2.97 ±0.31	91	87	101	93
		Control	$^0/_{19}$	+ 1.9	0.45 ±0.11	1.05 ±0.23	2.30 ±0.45	3.20 ±0.54	100	100	100	100
		LD_{10} = 430 mg kg^{-1}day^{-1}					LD_{50} = 565 mg kg^{-1}day^{-1}					

Appendix 9.5 Activity of derivatives of acetamide and urea against the M5 sarcoma (cont.)

COMPOUND	Schedule Days of injection	Dose $mgkg^{-1}$ day^{-1}	No. dead / Total	Mean body weight change (g)	Mean tumour volume ($cm^3 \pm$ s.d.) measured on days post implant				$\frac{T}{C}$ X 100%			
					DAY 12	DAY 16	DAY 20	DAY 24	DAY 12	DAY 16	DAY 20	DAY 24
N-METHYLACETAMIDE	1 - 17	1600	$^4/_5$	+2.7	0.29	0.54	1.27	2.81	85	62	72	100
		800	$^0/_5$	+2.8	0.27 ±0.08	0.74 ±0.16	1.53 ±0.23	2.81 ±0.47	79	85	87	100
		400	$^0/_5$	+2.7	0.26 ±0.10	0.76 ±0.18	1.67 ±0.34	2.84 ±0.47	76	87	95	101
		200	$^0/_5$	+2.4	0.30 ±0.11	0.83 ±0.17	1.66 ±0.05	2.98 ±0.41	88	95	94	106
		100	$^0/_5$	+2.4	0.35 ±0.06	0.95 ±0.15	1.65 ±0.20	3.02 ±0.44	103	109	94	107
		Control	$^0/_{18}$	+3.8	0.34 ±0.07	0.87 ±0.16	1.76 ±0.30	2.81 ±0.44	100	100	100	100
	LD_{10} = 880 $mgkg^{-1}day^{-1}$						LD_{50} = 1240 $mgkg^{-1}day^{-1}$					
N-METHYL-TRI-FLUOROMETHYLACETAMIDE	1 - 17	800	$^1/_5$	+2.7	0.17 ±0.09	0.53 ±0.06	1.71 ±0.36	2.48 ±0.47	53	76	101	95
		400	$^0/_5$	+4.0	0.35 ±0.15	0.80 ±0.12	1.84 ±0.29	2.54 ±0.25	109	114	108	97
		200	$^0/_5$	+4.3	0.31 ±0.12	0.56 ±0.14	1.56 ±0.13	2.58 ±0.23	97	80	92	98
		100	$^0/_5$	+3.9	0.33 ±0.01	0.67 ±0.21	1.71 ±0.36	2.52 ±0.32	103	96	101	96
		50	$^0/_5$	+3.4	0.32 ±0.12	0.72 ±0.25	1.85 ±0.41	2.87 ±0.63	100	103	109	110
		Control	$^0/_{18}$	+3.0	0.32 ±0.13	0.70 ±0.24	1.70 ±0.44	2.62 ±0.65	100	100	100	100
	$LD_{20} \sim$ 800 $mgkg^{-1}day^{-1}$											

Appendix 9.5 Activity of derivatives of acetamide and urea against the M5 sarcoma. (cont.)

COMPOUND	Dose mg kg^{-1}	Solvent	W.B.C. Count X $10^3 mm^{-3}$ ($\pm$ s.d.) on days post injection				
			Day 0	Day 2	Day 4	Day 6	Day 8
NMF	2300	Saline	7.5 $\pm$ 3.4	6.2 $\pm$ 2.0	10.2 $\pm$ 3.6	7.1 $\pm$ 1.1	9.1 $\pm$ 2.6
NMF	800	Saline	9.2 $\pm$ 3.2	10.4 $\pm$ 3.5	8.0 $\pm$ 2.4	8.5 $\pm$ 3.0	8.1 $\pm$ 2.7
Saline control	(0.1 ml)	Saline	8.6 $\pm$ 1.5	9.1 $\pm$ 2.5	8.5 $\pm$ 1.6	8.0 $\pm$ 2.2	9.3 $\pm$ 2.3
Cyclophosphamide	320	10% DMSO/OIL	8.1 $\pm$ 3.0	2.0 $\pm$ 1.5	0.7 $\pm$ 0.8	2.9 $\pm$ 2.2	4.5 $\pm$ 1.7
CCNU	40	10% DMSO/OIL	6.0 $\pm$ 2.4	3.9 $\pm$ 1.3	2.1 $\pm$ 1.1	3.9 $\pm$ 1.2	6.4 $\pm$ 1.6
10% DMSO/OIL Control	(0.1 ml)	10% DMSO/OIL	8.8 $\pm$ 3.6	10.7 $\pm$ 3.7	10.4 $\pm$ 3.7	9.7 $\pm$ 2.5	10.4 $\pm$ 5.0

Appendix 10.1 Peripheral white blood cell counts of mice (balb/c) treated with a single injection of NMF

COMPOUND	Dose mg kg^{-1}	Solvent	Platelet count X $10^5 mm^{-3}$ ($\pm$ s.d) on days post injection					
			Day 0	Day 2	Day 4	Day 6	Day 8	Day 10
NMF	2300	Saline	4.8 $\pm$ 0.7	4.8 $\pm$ 1.9	4.7 $\pm$ 1.4	5.0 $\pm$ 1.7	5.0 $\pm$ 1.6	7.8 $\pm$ 1.0
NMF	800	Saline	6.9 $\pm$ 2.5	5.7 $\pm$ 2.6	4.9 $\pm$ 2.0	4.8 $\pm$ 2.2	4.8 $\pm$ 1.4	5.4 $\pm$ 1.3
Saline Control	(0.1 ml)	Saline	4.6 $\pm$ 0.9	4.6 $\pm$ 1.8	4.9 $\pm$ 1.7	4.3 $\pm$ 1.7	5.5 $\pm$ 0.9	$\pm$
Cyclophosphamide	320	10% DMSO/OIL	5.8 $\pm$ 1.6	5.3 $\pm$ 2.1	5.3 $\pm$ 2.4	1.3 $\pm$ 0.5	1.2 $\pm$ 0.4	3.5 $\pm$ 1.9
CCNU	40	10% DMSO/OIL	4.4 $\pm$ 1.1	5.9 $\pm$ 2.2	7.6 $\pm$ 3.5	4.7 $\pm$ 2.7	3.4 $\pm$ 1.2	3.2 $\pm$ 1.1
10% DMSO/OIL Control		10% DMSO/OIL	5.3 $\pm$ 2.4	6.1 $\pm$ 2.3	4.0 $\pm$ 0.5	5.0 $\pm$ 2.5	5.0 $\pm$ 2.3	5.9 $\pm$ 3.5

Appendix 10.2 Peripheral platelet counts of mice (balb/c) treated with a single injection of NMF

Compound	Dose $mgkg^{-1}$	Solvent	Erythrocyte count X $10^6 mm^{-3}$ ($\pm$ s.d.) on day post injection				
			Day 0	Day 2	Day 4	Day 6	Day 8
NMF	2300	Saline	8.8 $\pm$ 0.8	9.4 $\pm$ 0.5	9.0 $\pm$ 1.1	8.7 $\pm$ 1.2	---
NMF	800	Saline	8.4 $\pm$ 0.8	8.7 $\pm$ 0.5	8.6 $\pm$ 0.6	9.1 $\pm$ 1.0	---
Saline control	(0.1ml)	Saline	8.1 $\pm$ 0.5	8.3 $\pm$ 0.8	8.4 $\pm$ 0.7	8.8 $\pm$ 0.9	---
Cyclophosphamide	320	10% DMSO/OIL	8.6 $\pm$ 0.6	8.8 $\pm$ 0.6	7.4 $\pm$ 1.0	8.7 $\pm$ 0.7	8.3 $\pm$ 0.8
CCNU	40	10% DMSO/OIL	8.9 $\pm$ 0.1	8.0 $\pm$ 0.3	7.0 $\pm$ 0.7	8.2 $\pm$ 0.5	7.6 $\pm$ 0.5
10% DMSO/OIL Control	(0.1ml)	10% DMSO/OIL	7.6 $\pm$ 0.5	8.2 $\pm$ 1.0	7.4 $\pm$ 0.4	7.9 $\pm$ 0.2	8.1 $\pm$ 1.5

Appendix 10.3 Peripheral erythrocyte counts of mice (balb/c) treated with a single injection of NMF

Compound	Schedule (days of injection)	Dose $mgkg^{-1}$ day^{-1}	Solvent	W.B.C. Counts X $10^3 mm^{-3}$ ($\pm$ s.d.) on days post injection					
				Day 0	Day 7	Day 14	Day 21	Day 28	Day 35
NMF	0	2300	Saline	8.6 $\pm$ 3.1	10.5 $\pm$ 1.1	7.1 $\pm$ 3.0	6.9 $\pm$ 3.4	6.7 $\pm$ 2.2	7.7 $\pm$ 2.0
NMF	0	800	Saline	8.2 $\pm$ 3.0	10.7 $\pm$ 2.7	10.9 $\pm$ 2.4	11.3 $\pm$ 4.7	7.8 $\pm$ 3.1	6.3 $\pm$ 1.1
NMF	0 - 4	400	Saline	8.1 $\pm$ 1.7	10.6 $\pm$ 2.6	6.0 $\pm$ 2.2	6.5 $\pm$ 2.9	5.0 $\pm$ 0.8	8.5 $\pm$ 1.9
NMF	0 - 4	200	Saline	7.9 $\pm$ 4.0	10.5 $\pm$ 1.8	8.8 $\pm$ 3.0	8.6 $\pm$ 2.4	6.8 $\pm$ 2.2	---
NMF	0 - 4	100	Saline	7.7 $\pm$ 1.5	11.4 $\pm$ 3.3	7.0 $\pm$ 1.9	10.0 $\pm$ 3.6	6.5 $\pm$ 3.9	---
Saline Control	0 - 4	(0.1ml)	Saline	6.4 $\pm$ 1.7	7.7 $\pm$ 2.7	9.8 $\pm$ 2.8	7.0 $\pm$ 2.5	6.4 $\pm$ 0.7	---
Cyclophosphamide	0	320	10% DMSO/OIL	5.5 $\pm$ 3.3	6.9 $\pm$ 1.4	10.6 $\pm$ 3.8	9.4 $\pm$ 2.8	7.5 $\pm$ 0.9	---
CCNU	0	40	10% DMSO/OIL	5.4 $\pm$ 2.2	7.8 $\pm$ 3.0	12.9 $\pm$ 3.4	7.7 $\pm$ 1.9	8.7 $\pm$ 0.4	---

Appendix 10.4 Long term study of the peripheral white blood cell counts of mice (balb/c) treated with NMF (Acute and chronic schedules)

Compound	Schedule (days of injection)	Dose $mgkg^{-1}day^{-1}$	Solvent	Platelet count X $10^5 mm^{-3}$ ($\pm$ s.d) on day post injection				
				Day 0	Day 7	Day 14	Day 21	Day 28
NMF	0 - 4	400	Saline	6.5 $\pm$ 2.5	5.3 $\pm$ 1.7	5.9 $\pm$ 1.3	6.6 $\pm$ 2.2	6.3 $\pm$ 1.2
NMF	0 - 4	200	Saline	6.1 $\pm$ 1.9	5.1 $\pm$ 2.4	6.4 $\pm$ 1.9	4.9 $\pm$ 1.5	5.7 $\pm$ 1.0
NMF	0 - 4	100	Saline	6.0 $\pm$ 2.0	7.0 $\pm$ 1.8	7.8 $\pm$ 2.9	5.7 $\pm$ 1.6	5.0 $\pm$ 0.6
Saline control	0 - 4	(0.1 ml)	Saline	5.0 $\pm$ 1.1	5.6 $\pm$ 1.8	5.0 $\pm$ 1.8	5.1 $\pm$ 1.2	5.8 $\pm$ 1.5
Cyclophosphamide	0	320	10% DMSO/OIL	5.8 $\pm$ 2.1	2.2 $\pm$ 1.5	5.6 $\pm$ 1.9	4.8 $\pm$ 1.4	4.9 $\pm$ 1.9
CCNU	0	40	10% DMSO/OIL	5.3 $\pm$ 2.0	2.0 $\pm$ 1.3	5.3 $\pm$ 1.9	4.4 $\pm$ 0.9	4.1 $\pm$ 1.7

Appendix 10.5 Long term study of the peripheral platelet counts of mice (balb/c) treated with NMF (chronic schedule)

Compound	Schedule (days of injection)	Dose $mgkg^{-1}$ day^{-1}	Solvent	Erythrocyte count X $10^6 mm^{-3}$ ($\pm$ s.d) on day post injection					
				Day 0	Day 7	Day 14	Day 21	Day 28	Day 35
NMF	0	2300	Saline	9.7 $\pm$ 0.9	10.4 $\pm$ 0.8	9.6 $\pm$ 1.1	8.9 $\pm$ 1.2	9.0 $\pm$ 0.9	8.3 $\pm$ 0.8
NMF	0	800	Saline	9.5 $\pm$ 0.8	10.3 $\pm$ 1.3	9.1 $\pm$ 0.8	10.1 $\pm$ 1.1	9.7 $\pm$ 1.3	7.7 $\pm$ 1.2
NMF	0 - 4	400	Saline	9.5 $\pm$ 1.2	8.5 $\pm$ 0.5	9.1 $\pm$ 0.9	9.6 $\pm$ 1.0	8.7 $\pm$ 0.9	7.8 $\pm$ 0.7
NMF	0 - 4	200	Saline	9.6 $\pm$ 0.2	12.0 $\pm$ 2.2	9.5 $\pm$ 1.0	10.1 $\pm$ 1.0	8.7 $\pm$ 0.5	---
NMF	0 - 4	100	Saline	9.1 $\pm$ 0.6	9.6 $\pm$ 0.9	8.6 $\pm$ 0.5	8.4 $\pm$ 0.6	8.4 $\pm$ 0.8	---
Saline Control	0 - 4	(0.1ml)	Saline	9.0 $\pm$ 0.5	8.7 $\pm$ 0.7	8.7 $\pm$ 0.6	---	---	---
Cyclophosphamide	0	320	10% DMSO/OIL	8.6 $\pm$ 0.6	8.7 $\pm$ 1.0	8.7 $\pm$ 0.6	7.9 $\pm$ 0.4	---	---
CCNU	0	40	10% DMSO/OIL	8.9 $\pm$ 0.1	---	8.5 $\pm$ 1.1	---	---	---

Appendix 10.6 Long term study of the peripheral erythrocyte counts of mice (balb/c) treated with NMF (acute and chronic schedules)

DOSE mg kg^{-1}	Plasma levels of SDH (units L^{-1} ± s.d.) at time points (h) post injection					
	3	6	12	24	48	72
1200	55±3	-	-	140±43	1156±166	204±11
800	39±14	43±26	40±6	1349±1019	1815±1614	244±265
400	32±3	37±5	29±2	32±10	21±5	24±2
200	29±2	27±6	39±5	29±5	15±2	26±3
Control	42±10	24±5	24±3	21±3	16±5	27±3

Appendix 10.7 Plasma levels of sorbitol dehydrogenase (SDH) in BDF_1 mice at various time points post injection of NMF.

DOSE (mg kg^{-1} day^{-1})	SCHEDULE (days)	Plasma levels of SDH (units L^{-1} ± s.d.) at time points post final injection (h)			
		3	24	48	72
400	1 - 10	87±39	365±365	29±0	37±32
200	1 - 10	43±13	24±8	26±3	21±2
Control	1 - 10	19±6	19±3	21±2	21±6

Appendix 10.8 Plasma levels of sorbitol dehydrogenase (SDH) in BDF_1 mice at various time points post final injection of NMF (chronic 10 day schedule).

DOSE (mg kg^{-1} day^{-1})	SCHEDULE (days)	Plasma levels of ALT (units L^{-1} ± s.d) at time points post final injection (h)			
		3	24	48	72
400	1 - 10	125±135	537±516	36±5	82±84
200	1 - 10	41±5	34±5	31±12	27±5
Control	1 - 10	27±7	34±5	34±2	34±7

Appendix 10.9 Plasma levels of alanine aminotransferase (ALT) in BDF_1 mice at various time points post final injection of NMF (chronic 10 day schedule).

DOSE (mg kg^{-1} day^{-1})	SCHEDULE (days)	Plasma levels of AST (units L^{-1} ± s.d.) at time points post final injection (h)			
		3	24	48	72
400	1 - 10	159±111	284±166	65±12	113±36
200	1 - 10	41±24	116±48	39±43	51±19
Control	1 - 10	70±29	101±46	82±17	60±24

Appendix 10.10 Plasma levels of aspartate aminotransferase (AST) in BDF_1 mice at various time points post final injection of NMF (chronic 10 day schedule).

CYCLOPHOSPHAMIDE $mgkg^{-1}day^{-1}$	NMF $mgkg^{-1}day^{-1}$	No. mice	W.B.C. ($\pm$ s.d.) X $10^{3}mm^{-3}$ measured on days post implant								
			DAY 12	DAY 14	DAY 16	DAY 18	DAY 20	DAY 22	DAY 24	DAY 26	DAY 28
320 day 12	-	10	13.7 $\pm$2.3	1.4 $\pm$0.6	0.4 $\pm$0.2	4.0 $\pm$0.9	9.9 $\pm$4.7	8.1 $\pm$1.4	11.6 $\pm$4.3	8.7 $\pm$2.9	5.9 $\pm$1.0
160 day 12	-	10	11.2 $\pm$1.8	2.6 $\pm$0.7	1.5 $\pm$0.7	4.7 $\pm$0.2	12.8 $\pm$3.7	9.6 $\pm$2.2	9.5 $\pm$1.4	10.2 $\pm$1.7	7.7 $\pm$1.8
160 days 12 and 15	-	10	8.5 $\pm$1.0	1.5 $\pm$0.1	0.8 $\pm$0.3	0.4 $\pm$0.2	2.2 $\pm$0.9	10.4 $\pm$2.0	10.9 $\pm$3.7	9.3 $\pm$1.4	6.0 $\pm$0.7
-	200 days 15 -24	10	14.5 $\pm$3.8	9.5 $\pm$2.2	7.5 $\pm$1.4	11.4 $\pm$4.0	8.7 $\pm$4.0	6.3 $\pm$3.3	14.3 $\pm$4.4	9.2 $\pm$1.2	7.9 $\pm$1.6
320 day 12	200 days 15 - 24	10	13.2 $\pm$2.1	1.4 $\pm$0.6	0.4 $\pm$0.2	3.8 $\pm$1.2	6.7 $\pm$0.6	8.4 $\pm$2.9	7.9 $\pm$1.1	8.1 $\pm$2.7	8.2 $\pm$3.1
160 day 12	200 days 15 - 24	10	7.3 $\pm$1.2	2.1 $\pm$0.6	1.2 $\pm$0.7	5.7 $\pm$2.5	11.5 $\pm$1.3	8.0 $\pm$2.0	10.9 $\pm$1.8	11.2 $\pm$2.7	7.7 $\pm$1.7
-	-	10	7.6 $\pm$2.8	10.5 $\pm$2.4	8.3 $\pm$0.8	9.5 $\pm$1.6	9.3 $\pm$1.6	9.8 $\pm$2.4	12.7 $\pm$3.1	10.0 $\pm$2.1	6.8 $\pm$1.8

Appendix 11.1 Peripheral white blood cell counts (W.B.C. $\pm$ s.d.) of M5 sarcoma-bearing mice treated with the Cyclophosphamide/NMF combination

CYCLOPHOSPHAMIDE $mgkg^{-1}day^{-1}$	NMF $mgkg^{-1}day^{-1}$	No. mice	Tumour volumes (cm^3 ± s.d.) measured on days post implant										
			DAY 12	DAY 16	DAY 20	DAY 24	DAY 28	DAY 32	DAY 36	DAY 40	DAY 44	DAY 48	DAY 52
320 day 12	-	10	0.7 ±0.1	0.4 ±0.2	0.5 ±0.1	0.3 ±0.2	0.2 ±0.2	0.4 ±0.4	1.3 ±0.8	3.4 ±0.7	5.3 ±1.2	6.8 ±1.4	-
160 day 12	-	10	0.9 ±0.2	0.6 ±0.2	0.7 ±0.1	0.5 ±0.2	0.7 ±0.4	2.2 ±1.1	3.6 ±0.9	5.8 ±0.9	-	-	-
160 days 12 and 15	-	10	0.9 ±0.3	0.6 ±0.1	0.4 ±0.1	0.4 ±0.2	0.3 ±0.2	0.3 ±0.3	0.9 ±0.7	2.4 ±1.1	4.4 ±1.8	5.9 ±1.7	-
-	200 days 15 - 24	10	0.8 ±0.3	1.3 ±0.3	0.7 ±0.1	0.3 ±0.2	NM	1.8 ±0.4	4.3 ±0.7	6.0 ±1.2	-	-	-
320 day 12	200 days 15 - 24	10	0.8 ±0.1	0.3 ±0.2	0.2 ±0.1	NM	NM	NM	NM	NM	1.2 ±0.4	2.3 ±0.8	5.0 ±1.3
160 day 12	200 days 15 - 24	10	0.9 ±0.1	0.5 ±0.1	0.3 ±0.1	NM	NM	NM	NM	0.4 ±0.3	1.7 ±0.7	4.5 ±0.8	-
-	-	10	0.8 ±0.2	2.1 ±0.3	3.5 ±0.7	5.5 ±1.0	7.2 ±0.8	-	-	-	-	-	-

Appendix 11.2 Mean tumour volumes of M5 sarcoma-bearing mice treated with the Cyclophosphamide/NMF combination

CYCLOPHOSPHAMIDE $mgkg^{-1}day^{-1}$	NMF $mgkg^{-1}day^{-1}$	No. Mice	Platelets ($\pm$ s.d.) X $10^5 mm^{-3}$ measured on days post implant							
			DAY 13	DAY 15	DAY 17	DAY 19	DAY 21	DAY 23	DAY 25	DAY 27
320 day 12	-	10	6.5 $\pm$2.0	6.8 $\pm$2.2	8.3 $\pm$1.3	7.0 $\pm$2.7	-	-	-	-
160 day 12	-	10	8.2 $\pm$1.6	8.9 $\pm$1.9	8.3 $\pm$2.4	5.3 $\pm$0.5	-	-	-	-
160 days 12 and 15	-	10	6.6 $\pm$2.4	9.2 $\pm$3.8	9.2 $\pm$3.5	6.8 $\pm$1.2	4.9 $\pm$2.6	7.3 $\pm$1.2	9.8 $\pm$2.1	11.3 $\pm$3.1
-	200 days 15 - 24	10	8.6 $\pm$2.6	9.2 $\pm$1.5	7.8 $\pm$1.1	6.4 $\pm$1.3	12.0 $\pm$3.0	6.7 $\pm$2.3	7.5 $\pm$1.6	9.2 $\pm$1.4
320 day 12	200 days 15 - 24	10	7.3 $\pm$0.8	7.1 $\pm$1.4	6.3 $\pm$0.5	7.2 $\pm$0.6	10.4 $\pm$2.7	11.7 $\pm$4.9	6.6 $\pm$1.3	7.8 $\pm$2.8
160 day 12	200 days 15 - 24	10	6.2 $\pm$1.0	6.8 $\pm$1.2	7.4 $\pm$2.1	7.3 $\pm$2.1	-	-	-	-
-	-	10	5.9 $\pm$1.3	7.1 $\pm$1.9	6.9 $\pm$0.6	6.9 $\pm$1.6	6.7 $\pm$1.2	6.5 $\pm$0.4	6.5 $\pm$0.6	7.2 $\pm$2.1

Appendix 11.3 Peripheral platelet counts ($\pm$ s.d.) of M5 sarcoma bearing mice treated with the Cyclophosphamide/NMF combination

CYCLOPHOSPHAMIDE $mgkg^{-1}$ DAY 0	NMF $mgkg^{-1}day^{-1}$ DAYS 3 - 12	Plasma levels of SDH (units $L^{-1} \pm$ s.d.) at time points (h) post final injection			
		3	24	48	72
320	-	26 ± 3	88 ± 82	24 ± 3	-
160	-	24 ± 5	35 ± 21	24 ± 5	-
320	200	19 ± 6	19 ± 5	19 ± 6	19 ± 4
160	200	26 ± 8	34 ± 18	18 ± 3	14 ± 2
-	-	26 ± 1	21 ± 3	23 ± 4	16 ± 2

Appendix 11.4a Plasma levels of sorbitol dehydrogenase (SDH) in BDF_1 mice at various time points post final injection in the Cyclophosphamide/NMF combination experiment (cf. appendix 10.8).

CYCLOPHOSPHAMIDE $mgkg^{-1}$ DAY 0	NMF $mgkg^{-1}day^{-1}$ DAYS 3 - 12	Plasma levels of ALT (units $L^{-1} \pm$ s.d.) at time points (h) post final injection		
		3	24	48
320	-	51 ± 2	104 ± 89	27 ± 5
160	-	39 ± 7	53 ± 22	36 ± 14
320	200	24 ± 5	22	14 ± 2
160	200	22 ± 2	22 ± 7	12 ± 2
-	-	35 ± 17	27 ± 5	25 ± 6

Appendix 11.4b Plasma levels of alanine aminotransferase (ALT) in BDF_1 mice at various time points post final injection in the Cyclophosphamide/NMF combination experiment (cf. appendix 10.9)

CYCLOPHOSPHAMIDE $mgkg^{-1}$ DAY 0	NMF $mgkg^{-1}day^{-1}$ DAYS 3 - 12	Plasma levels of AST (units L^{-1} ± s.d.) at time points (h) post final injection		
		3	24	48
320	-	166 ± 7	125	92 ± 17
160	-	123 ± 27	159 ± 77	157 ± 46
320	200	77 ± 12	101 ± 53	48 ± 14
160	200	53 ± 12	80 ± 5	67 ± 24
-	-	131 ± 84	100 ± 33	115 ± 73

Appendix 11.4c Plasma levels of aspartate aminotransferase (AST) in BDF_1 mice at various time points post final injection in the Cyclophosphamide/ NMF combination experiment (appendix 10.10)

	NMF dose mg kg^{-1} day^{-1}	HMM dose mg kg^{-1} day^{-1}	No. dead / Total	Mean body weight change (g)	MEAN TUMOUR VOLUME (cm^3 ± s.d.)										
					DAY 12	DAY 16	DAY 20	DAY 24	DAY 28	DAY 32	DAY 36	DAY 40	DAY 44	DAY 48	DAY 52
A	400		1/5	- 4.5	NM	NM	NM	NM	NM	NM	0.31 ±0.21	1.73 ±0.68	3.84 ±0.23	6.67 ±0.38	-
B	300		0/5	- 0.9	NM	NM	NM	NM	NM	0.82 ±0.23	2.21 ±0.86	3.55 ±1.10	5.92 ±0.62	-	-
C	200		0/5	+ 1.5	NM	NM	NM	NM	0.76 ±0.01	2.15 ±0.10	3.75 ±1.47	5.67 ±1.77	-	-	-
D	100		0/5	+ 2.2	NM	NM	0.18 ±0.13	1.57 ±0.22	2.77 ±0.59	4.63 ±1.51	6.96 ±1.65	9.68 ±2.43	-	-	-
E	50		0/5	+ 2.6	NM	0.42 ±0.36	1.04 ±0.44	3.12 ±0.97	4.87 ±1.20	6.82 ±1.51	8.65 ±1.48	-	-	-	-
F		150	5/5	-	-	-	-	-	-	-	-	-	-	-	-
G		100	0/5	+ 0.5	NM	NM	NM	NM	1.03 ±0.27	2.21 ±0.33	3.24 ±0.49	5.16 ±0.74	7.00 ±0.64	9.41 ±0.70	-
H		50	0/5	+ 2.7	NM	NM	0.64 ±0.20	1.64 ±0.43	2.91 ±0.49	4.82 ±0.66	5.94 ±0.58	8.47 ±1.43	-	-	-
I		25	0/5	+ 2.9	0.17 ±0.09	1.44 ±0.14	2.37 ±0.43	4.13 ±0.66	6.21 ±1.06	7.71 ±1.40	11.21 ±1.70	-	-	-	-
J	300	50	0/5	+ 1.2	NM	NM	NM	NM	NM	NM	0.29 ±0.36	1.73 ±0.48	2.68 ±0.81	4.40 ±0.11	5.72 ±0.92
K	200	100	0/5	+ 0.4	NM	NM	NM	NM	NM	NM	0.62 ±0.46	1.72 ±0.94	3.47 ±0.98	5.23 ±0.11	6.76 ±1.63
L	200	50	0/5	+ 1.9	NM	NM	NM	NM	NM	NM	1.15 ±0.73	2.30 ±1.24	4.10 ±1.24	6.46 ±1.42	-
M	100	100	0/5	+ 0.8	NM	NM	NM	NM	NM	0.80 ±0.43	2.27 ±0.99	3.82 ±0.68	5.78 ±1.15	7.77 ±1.90	-
N	100	50	0/5	+ 1.0	NM	NM	NM	NM	0.57 ±0.11	1.81 ±0.66	4.71 ±0.69	6.05 ±1.18	-	-	-
O	50	100	0/5	+ 1.0	NM	NM	NM	NM	0.86 ±0.32	2.18 ±0.47	3.79 ±1.06	5.61 ±1.25	-	-	-
P	50	50	0/5	+ 2.1	NM	NM	NM	0.54 ±0.39	1.56 ±0.41	3.13 ±0.58	4.58 ±1.05	7.05 ±1.07	-	-	-
Control			0/9	+ 3.6	0.84 ±0.15	2.35 ±0.39	3.87 ±0.56	5.44 ±0.69	7.33 ±1.30	9.82 ±1.78	11.49 ±2.38	-	-	-	-

Appendix 11.5 HMM/NMF Combination - mean tumour volumes

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