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IN VITRO STUDIES WITH HEXAMETHYLMELAMINE

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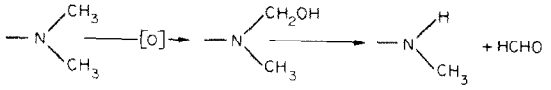
Abstract—Hexamethylmelamine (HMM) is effective against a number of human cancers. Studies using mouse lymphoma cells *in vitro* have shown that HMM is quite non-toxic but is converted by liver microsomes to cytotoxic products. A correlation was found between the ability of analogues of HMM to be demethylated and their antitumour activity. Experiments to test whether the cytotoxicity occurring after microsomal metabolism of HMM was due to formation of either a methylol or fomaldehyde were equivocal. Some water soluble derivatives of HMM were also shown to have antitumour activity.

Hexamethylmelamine (HMM) a cytotoxic *S*-triazine has been evaluated in extensive clinical trials [1–3] and has proved to be an effective antitumour agent [4–8]. Despite its effectiveness against human cancer, HMM is inactive against most commonly used animal tumour systems making difficult a study of its mechanism of action. It has, for example, no activity against L1210 leukaemia, marginal activity against Sarcoma 180 [9] and slight but significant activity against the Walker carcinoma 256 [10]. However, it has recently been shown to be active against a mouse plasma cell tumour (PC6) [11]. This tumour undergoes complete regression when treated with HMM, even when established, and hence may be used as an animal model for further investigation of this drug.

The metabolism of HMM has been studied in several species [12, 13, 15, 16] and *N*-demethylation to lower methylmelamines appears to be the major pathway for metabolism *in vivo* [18]. Worzalla has shown that HMM can be *N*-demethylated by hepatic microsomes [17] and that measurement of formaldehyde release may be used as an index of metabolism *in vitro*, while De Milo and Borkovec report that oxidative demethylation of HMM involves elimination of formaldehyde from methylol intermediates [15].



It is not known whether HMM is itself the tumour active agent, or whether it requires conversion to an active metabolite. However, since melamine and various methylmelamines are the only major metabolites, Worzalla [18] has concluded that either HMM itself, an *N*-demethylated metabolite, or an unidentified intermediate, such as a methylol must represent the active form of the drug. Since all methylmelamines undergo metabolism similar to HMM, then further testing of these analogues against a sensitive animal tumour might uncover agents which have more favourable anti-tumour properties than HMM, for example, increased selectivity of action or greater water solubility.

The mechanism of action of HMM is unknown. Although it has no alkylating activity *per se*, it has a spectrum of therapeutic activity and toxicity resembling that of the alkylating agents. It has been proposed [14] that HMM may act by release of formaldehyde and that the role of the methyl group in HMM is to act as sources of formaldehyde, a known weak alkylating agent. Alternatively, the proposed reactive intermediates, the methylols, might act by a mechanism analogous to alkylation. It is of interest that Heere and Donnelly [19] have found, using Ehrlich ascites cells, that HMM exhibited greater inhibition of uptake of DNA and RNA precursors than of protein precursors since many alkylating agents have greater effects on nucleic acid synthesis than on protein synthesis. Borkovec and De Milo [14] have suggested that HMM may alternatively function as a pyrimidine anti-metabolite.

The present paper reports a study of the structural activity relationship of a series of substituted melamines, including a number of water soluble derivatives. Studies have been carried out *in vitro* in order to determine how these compounds are metabolised, and to try to identify the active metabolite of HMM. The relative importance of methyols and formaldehyde as cytotoxic agents has been examined in some detail.

MATERIALS AND METHODS

Chemicals. Unlabelled HMM was provided by the National Cancer Institute, Bethesda.

Other methylmelamines were synthesized either by Dr. D. E. V. Wilman or Professor W. C. J. Ross of the Chester Beatty Research Institute.

HMM-ring-^{14}C was synthesized by Dr. Nguyen-Hoang-Nam of the Service des Molécules Marquées, C.E.N.-Saclay,B.P. No. 2, Gif-sur-Yvette 91, France.

(i) *Antitumour testing.* The ADJ/PC6 plasma cell tumour was transplanted in female BALB/c mice by subcutaneous implantation of tumour fragments (approx. 1 mm cube) under sterile conditions. Drug effectiveness was measured by comparison of tumour

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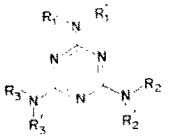
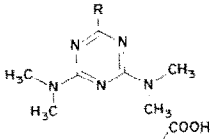
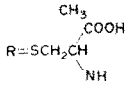
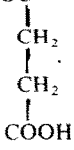
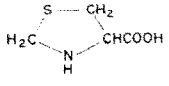
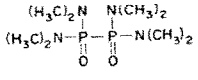
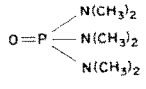
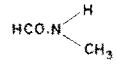
Table 1. Structure activity relationships



	R_1	R'_1	R_2	R'_2	R_3	R'_3
1.	$-CH_3$	$-CH_3$	$-CH_3$	$-CH_3$	$-CH_3$	$-CH_3$
2.	$-H$	$-CH_3$	$-CH_3$	$-CH_3$	$-CH_3$	$-CH_3$
3.	$-H$	$-CH_3$	$-H$	$-CH_3$	$-CH_3$	$-CH_3$
4.	$-H$	$-H$	$-CH_3$	$-CH_3$	$-CH_3$	$-CH_3$
5.	$-H$	$-CH_3$	$-H$	$-CH_3$	$-H$	$-CH_3$
6.	$-H$	$-H$	$-H$	$-H$	$-CH_3$	$-CH_3$
7.	$-H$	$-H$	$-H$	$-H$	$-H$	$-CH_3$
8.	$-H$	$-H$	$-H$	$-H$	$-H$	$-H$
9.			R = Cl			
10.						
11.	$-C_2H_5$	$-C_2H_5$	$-CH_3$	$-CH_3$	$-CH_3$	$-CH_3$
12.	$-H$	$-CH_3$	$-C_2H_5$	$-C_2H_5$	$-C_2H_5$	$-C_2H_5$
13.	$-CH_2CH_2OH$	$-CH_2CH_2OH$	$-CH_3$	$-CH_3$	$-CH_3$	$-CH_3$
14.	$-CH_3$	$-CH_2CH_2OH$	$-CH_3$	$-CH_3$	$-CH_3$	$-CH_3$
15.	$-CH_3$	$-CH_2COOH$	$-CH_3$	$-CH_3$	$-CH_3$	$-CH_3$
16.	$-CH_3$	$-CH_2CH_2OOC$ 	$-CH_3$	$-CH_3$	$-CH_3$	$-CH_3$
17.	$-CD_3$	$-CD_3$	$-CD_3$	$-CD_3$	$-CD_3$	$-CD_3$
18.	$-CH_3$	$-CH_2OH$	$-CH_3$	$-CH_3$	$-CH_3$	$-CH_3$
19.	$-CH_3$	$-CH_2OH$	$-CH_3$	$-CH_2OH$	$-CH_3$	$-CH_2OH$
20.	$-C_2H_5$	$-C_2H_5$	$-C_2H_5$	$-C_2H_5$	$-C_2H_5$	$-C_2H_5$
21.						
22.						
23.						
24.						

* ± Nicotinamide.
† Expressed as % demethylation of one methyl group.

of the methylmelamines

†Degree of demethylation	Relative degree demethylation (HMM = 1)	LD_{50} mg/kg	ID_{90} mg/kg	T.I. PC6
− Nicot* = 20.3%	1.0	113	11	10.3
+ Nicot = 70.9%				
− Nicot = 15.1%	0.54	95	41	2.3
+ Nicot = 38.4%	0.74			
− Nicot = 5.0%	0.24	155	86	1.8
+ Nicot = 10.9%	0.15			
− Nicot = 4.5%	0.21	112	29	3.9
+ Nicot = 15.2%	0.21			
− Nicot = 0.57%	0.028	56	—	Inactive
+ Nicot = 1.83%	0.026			
− Nicot = 0.58%	0.028	270	—	Inactive
+ Nicot = 0.96%	0.014			
− Nicot = 0.0%	0.0	270	—	Inactive
+ Nicot = 0.77%	0.01			
—	—	112	—	Inactive
+ Nicot = 21.5%	0.80	190	—	Inactive
+ Nicot = 2.39%	0.03	380	—	Inactive
+ Nicot = 74.7%	0.99	380	155	2.5
+ Nicot = 8.87%	0.12	283	—	Inactive
− Nicot = 10.6%	0.35	56.5	—	Inactive
+ Nicot = 22.7%	0.46			
+ Nicot = 14.5%	0.54	450	90	5.7
+ Nicot = 0.38%	0.01	225	—	Inactive
+ Nicot = 8.8%	0.33	450	205	2.2
+ Nicot = 51.2%	1.05	112	19	5.9
	1.0			
> 100%	> 1	112	38.5	2.9
> 100%	> 1	112	39	2.9
%De-ethylation	—	—	—	Inactive
+ Nicot = 17.4%				
—	—	112	—	Inactive
+ Nicot = 80%	0.16	2.8	—	Inactive
+ Nicot = 10%	0.25	1120	480	2.3
+ Nicot = 0%	—	1120		Inactive

weights of treated with control groups. The drugs were given as sonicated suspensions either in oil, 10% DMA/oil or acetone/oil, as five daily intraperitoneal (i.p.) injections. Treatment was started 24 days after transplant when tumours were well established, and the animals were sacrificed and tumours weighed 10 days after the first injection. Several dose levels of the drugs were used ranging from tumour non-effective to lethal, thus enabling the calculation of a therapeutic index [20].

(ii) N-*Demethylation by liver microsomes.* This involved the use of 'washed' liver microsomes prepared by differential centrifugation of rat liver homogenate. Microsomes were prepared from the livers of rats given water containing sodium phenobarbitone (500 mg/l) for 7 days.

Livers were removed and, after rinsing in ice-cold isotonic KCl, homogenized in KCl (1 g/4 ml KCl) at 0° in a Teflon–glass homogenizer. The resultant homogenate was then centrifuged at 10,000 *g* and 0° for 20 min and the supernatant decanted. Microsomes were prepared from this supernatant by centrifugation at 100,000 *g* for 1 hr at 4°. The microsomal pellet was then resuspended in KCl and recentrifuged at 100,000 *g* to give a 'washed' preparation.

To measure de-alkylation, the microsomes were suspended in 0.1 M KPO_4 buffer pH 7.4 to produce a suspension containing 1 g liver wet weight in each 4 ml. The reaction mixture consisted of 3 ml microsomal suspension together with the following cofactors in 1 ml phosphate buffer.

1.5 μm NADP, 100 μm Nicotinamide, 35 μm G6P, 25 μm $MgCl_2$ and 1 μl G6P dehydrogenase (Boehringer). 1 ml of a solution of semicarbazide HCl (1.11 mg/ml) in KPO_4 buffer was added to the reaction mixture to trap the aldehyde release during metabolism.

The substrate concentration was 5 μm (0.1 ml) dissolved in dimethylsulphoxide (DMSO) and the volume of incubation mixture was made up to 8.0 ml with buffer. Incubation was carried out at 37° for 1 hr with shaking to ensure maximum metabolism. A blank reaction mixture without substrate is employed in each experiment to correct for endogenous aldehyde formation. The reaction was terminated by the addition of 2 ml 5% $ZnSO_4$ + 2 ml saturated $Ba(OH)_2$, and the precipitate thus produced was removed by centrifugation.

To measure demethylation, 5 ml aliquots of supernatant were reacted with 2 ml Nash reagent, and the coloured complex assayed for formaldehyde by the method of Cochin and Axelrod [21].

To measure de-ethylation of hexaethylmelamine (HEM), the supernatant was first distilled; 1 ml distillate was treated with *p*-hydroxybiphenyl reagent, and acetaldehyde estimated colorimetrically according to the method of Stotz [22].

(iii) *Isolation and identification of metabolites.* Following incubation of drug (0.1 ml) with microsomes (10 ml) and cofactors (1 ml), as described above, protein was precipitated with three volumes of ethanol and the latter removed by evaporation. The aqueous suspension of metabolites remaining was extracted three times with 10 ml aliquots of chloroform, the extract evaporated to dryness, and the remaining solid redissolved in 0.5 ml chloroform. Thirty μl of this solution was applied to a thin-layer plate containing silica gel (3 mm thickness) and chromatographed using either ethyl acetate or ether/H_2O as solvent. The chromatogram was finally examined under u.v. light. The identity of the metabolites was elucidated by comparison with suitable standards which were chromatographed alongside the microsomal extract.

Where ^{14}C-ring labelled HMM was used, the chromatogram was scanned for radioactive areas using a Panax Radiochromatogram Scanner.

(iv) *Bioassay.* The sensitivity of tumour cells to HMM and its derivatives was determined using the bioassay procedure of Connors *et al.* [23]. In these experiments a bioassay system involving the TLX/5 lymphoma was used.

TLX/5 ascites cells were obtained from tumour-bearing CBA/LAC mice, washed in sterile isotonic saline, and suspended in TC 199/horse serum (60:40) to give a final cell concentration of 1.3×10^6 cells/ml. Cells were treated with various concentrations of the drug under test in DMSO for 2 hr at 37°, a non-treated control being included. The resulting mixture was then injected i.p. (0.1 ml per animal) into groups of 5 mice. The subsequent tumour-induced deaths were recorded and the results expressed as a percentage increase in survival time (I.S.T.).

For those drugs requiring liver activation, this was reproduced *in vitro* by the addition of microsomes and cofactors (as before) to the incubate of drug and tumour cells.

In 'protection' experiments where the aim was to protect against formaldehyde toxicity semicarbazide (1000 μg/ml) was added 30 min prior to the addition of the drug to minimize its cytotoxic effects.

RESULTS

(i) *Structure activity relationships.* The results obtained from a study of the structure-activity relationships of the melamine derivatives are summarized in Table 1. There appears to be a definite correlation between the degree of demethylation *in vitro* and their antitumour activity. The ability of the methylmelamines to undergo demethylation is seen to decrease with the number of methyl groups in the molecule, and there is a parallel decrease in therapeutic index.

Hexaethylmelamine (HEM) undergoes de-ethylation to a much smaller extent (17.4%) than the corresponding hexamethyl compound is demethylated (65%).

In general, the water soluble derivatives do not readily demethylate and are correspondingly poor antitumour agents. One exception is the chloro compound (Structure 9), which is a good demethylator but is totally inactive in the test system employed. The hemisuccinate of carboxy HMM (Structure 16) does demethylate to some extent, comparable to TMM, and has slight but significant antitumour activity. DHE TMM (Structure 13) also shows fairly good demethylating ability, but this compound is quite inactive. It is also, however, very toxic to mice (LD_{50} 56.5 mg/kg). The corresponding monohydroxy derivative (Structure 14) is of interest since it is very much less toxic (LD_{50} = 450 mg/kg), is a good demethylator, and has good antitumour activity.

The two methylols, pentamethylmonomethylolme-

Table 2. Activation of HMM at various dose levels and effect of semicarbazide on cytotoxicity

Concentration of drug (μg/ml)	Survival time (days)	Mean	% I.S.T.
Control	10, 11, 11, 11, 11	10.8	—
1000 Semicarbazide (control)	11, 11, 11, 11, 11	11.0	—
1000 HMM	10, 11, 11, 11, 11	10.8	—
1000 HMM + micro.	15, 15, 15, 16, 17	15.6	44.4
500 HMM + micro.	13, 13, 14, 14, 15	13.8	27.8
250 HMM + micro.	13, 13, 14, 14, 16	14.0	29.6
1000 HMM + micro. + semicarbazide	11, 11, 11, 11, 11	11.0	1.85

lamine (Structure 18) and trimethyltrimethylol melamine (Structure 19) have only shown a small degree of activity against the PC6 tumour, compared with HMM, and their therapeutic indices are the same. These compounds break down spontaneously under the demethylation assay conditions employed (heat at 60° for 30 min for Nash reaction) with release of formaldehyde. Trimethylol melamine, however, is approx. 100 times more water soluble than HMM.

The demethylation of related compounds has also been looked at, e.g. hexamethylphosphoramide (HMP) (Structure 23). This compound undergoes demethylation to approximately the same extent as tetramethylmelamine (TMM), and has a similar therapeutic index. Schradan (octamethylpyrophosphoramide) (Structure 22) shows little or no ability to demethylate and is inactive in the PC6 test system. Thioproline (Structure 21), a known formaldehyde releaser, has also proved to be completely inactive.

Demethylation studies in the presence and absence of nicotinamide have further shown that the presence of this cofactor considerably enhances the metabolism of HMM and its analogues. With the parent compound, e.g. the metabolism increases three-fold when nicotinamide is present in the metabolism mixture, and with PMM and TMM derivatives, at least a two-fold increase is observed.

(ii) *Thin-layer chromatographic analysis.* Thin-layer chromatographic analysis of the microsomal metabolism extract revealed the presence of two metabolites of HMM. These have been identified by comparison with appropriate reference compounds as pentamethylmelamine (PMM) and tetramethylmelamine (TMM), and the identity of the major metabolite, i.e. PMM, has been verified by mass spectrometry. Similarly, when PMM was demethylated, a single spot was observed, corresponding to N^2, N^2, N^4, N^6-TMM, but no further metabolism of either of the two isomeric TMMs was apparent.

When HEM was looked at in this system, no metabolites were detected. Using [^{14}C]HMM, three peaks of radioactivity were detected corresponding to the two metabolites and HMM itself. When nicotinamide was present the HMM peak was greatly reduced and two large peaks appeared corresponding to PMM and TMM, the second larger than the first. In the absence of nicotinamide, however, the HMM peak is much larger while that of PMM is slightly lower and the TMM peak is small.

(iii) *Bioassay.* Using optimum conditions for metabolism it has been possible to activate HMM in the bioassay system which uses TLX/5 lymphoma cells (Table 2). From the table it can be seen that a concentration of 1000 μg/ml of HMM has no effect on the lymphoma cells as judged by their ability to grow after injection to mice. However, in the presence of microsomes, a concentration of 1000 μg/ml gave an I.S.T. of 44 per cent implying a large cell kill (Table 2). However, since this could be due not only to the activation of HMM to a reactive metabolite, but also to the generation of formaldehyde, an experiment was carried out in which HMM was activated *in vitro*, but in the presence of semicarbazide which should react with any formaldehyde produced and prevent cytotoxicity. Table 2 shows that when semicarbazide is added the activation of HMM by microsomes is more or less completely prevented. It has been shown that formaldehyde is cytotoxic at a concentration of about 12.5 μg/ml (Table 3) and that this effect can be abolished by semicarbazide, since even at the supralethal concentration of 100 μg/ml formaldehyde if semicarbazide is present there is no detectable cytotoxicity (Table 3). However, pentamethylmonomethylol melamine (Structure 18) not only showed good activity in the bioassay without the need for microsomal activation (Table 3), but also the cytotoxicity could not be reduced by semicarbazide. Thioproline also showed some activity when tested in the bioassay but its effects were reduced by semicarbazide (Table 3).

Neither pentamethylmelamine nor the two tetramethylmelamines had any activity when bioassayed in the absence of microsomes.

Table 3.

Treatment	Non-toxic concentration (μg/ml)	Concentration to kill Ca 90% cells (μg/ml)	Supralethal concentration
Formaldehyde	6.25	21.5	100
Formaldehyde + semicarbazide	100		
CB 10–369	23.4	93.7	375
CB 10-369 + semicarbazide			375
Thioproline	250	1000	
Thioproline + semicarbazide	1000		

DISCUSSION

From the *in vitro* studies there appears to be a relationship in the methylmelamine series between amount of demethylation and activity against the PC6 plasma cell tumour *in vivo*. The suggestion is that activation by demethylation is an important prerequisite for antitumour activity since hexamethylmelamine for example, which has a good therapeutic index on the plasma cell tumour, is readily demethylated while dimethylmelamine, which is not demethylated, is devoid of anti-tumour activity. The active intermediate could conceivably be a methylol which could condense with the amino groups of proteins and nucleic acids, or formaldehyde which is mutagenic probably as a result of interaction with DNA [24]. The bioassay results however are equivocal in this respect. While hexamethylmelamine clearly requires microsomal activation before it causes cytotoxicity, this effect can be greatly reduced by the presence of semicarbazide in the medium. Since it has also been shown that the cytotoxicity of formaldehyde can be prevented by semicarbazide the implication is that the effects of hexamethylmelamine are mediated by release of formaldehyde. The postulated primary metabolite of hexamethylmelamine, namely monomethylolpentamethyl melamine (Structure 18, Table 3) is, as expected, cytotoxic to cells in culture without the need for microsomal activation. However, in this case the cytotoxicity cannot be prevented by semicarbazide implying that under the conditions of the bioassay there is no cytotoxic level of formaldehyde produced. Either hexamethylmelamine does not produce formaldehyde via a monomethylol, which is unlikely, or there must be some pharmacokinetic explanation of these findings. The pentamethyl monomethylol for instance, when added to cells in a high concentration (as in the bioassay), may readily be taken up by lymphoma cells. However, where the same compound is being generated by activation of hexamethylmelamine, the concentration may never be high and break down to formaldehyde may take place to a large extent extracellularly. It has in fact been recently shown that it is possible to obtain some protection against the cytotoxicity of these methylols with semicarbazide, if the trapping agent and drug are added simultaneously, that is without the normal preincubation period.

It has also been found that if sufficiently large amounts of liver microsomes are used in an attempt to increase metabolism of HMM, the DMSO used as solvent is itself demethylated, and the formaldehyde thus produced can cause cytotoxicity. This is indicative of a high degree of sensitivity of TLX/5 cells *in vitro* to the cytotoxic agent formaldehyde.

The low activity of the methylol *in vivo* may again be a feature of its half life and rate of breakdown to formaldehyde compared to hexamethylmelamine. However, even if the active metabolite of hexamethylmelamine is the monomethylol, it is difficult to see how the latter has anti-tumour selectivity if it merely acts as a releaser of formaldehyde, especially as other known formaldehyde releasers have no activity against the PC6 tumour (Table 1). It could be that *in vivo* hexamethylmelamine gives rise to a low but prolonged serum concentration of formaldehyde compared with other formaldehyde releasers. Alternatively, the methylol might act as a membrane transport form of formaldehyde and break down to a greater extent in the more acid environment of tumour cells. It is also of interest that certain types of tumour cells have been shown to have low levels of the enzyme N^5-methyltetrahydrofolate homocysteine methyl transferase [25], and unlike normal cells are unable to convert 5-methyltetrahydrofolate to tetrahydrofolic acid. It is a possibility that formaldehyde may be reacting with tetrahydofolate to cause a cellular deficiency of this essential cofactor which may be replaced in normal cells by conversion of 5-methyltetrahydrofolate but not in tumour cells.

Hexamethylmelamine is of particular interest because it has only recently been shown to be active against human tumours of the lung and kidney growing in mice deprived of T lymphocytes. The finding that certain water soluble derivatives of hexamethylmelamine have anti-tumour activity is of interest since hexamethylmelamine itself is very insoluble and cannot be administered clinically in parenteral form.

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