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STUDIES OF THE MODE OF ACTION OF ANTITUMOUR TRIAZENES AND TRIAZINES—V. THE CORRELATION OF THE *IN VITRO* CYTOTOXICITY AND *IN VIVO* ANTITUMOUR ACTIVITY OF HEXAMETHYLMELAMINE ANALOGUES WITH THEIR METABOLISM*

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Abstract—Experiments were conducted to ascertain whether the antitumour activity of hexamethylmelamine analogues correlated with their *in vitro* cytotoxicity and metabolism. Two analogues, namely pentamethylmelamine (PMM) and 2,2,4,4-tetramethylmelamine (TMM), and hexamethylmelamine (HMM) itself were shown to be active towards the murine ADJ/PC6A (PC6) plasmacytoma; another three, 2-chloro-4,6-bis(dimethylamino)-1,3,5-triazine (CBDT), 2,4-bis-(dimethylamino)-6-hydrazino-1, 3,5-triazine (HBDT) and 2,4,6-trimethylmelamine (TriMM) were inactive against the same tumour. The cytotoxicity of these compounds was examined against a PC6 tumour cell line *in vitro*. In the absence of liver microsomal activation only CBDT proved to be significantly cytotoxic at a concentration of 5 mM. In the presence of murine liver microsomes the three active antitumour agents were all cytotoxic at this concentration whereas HBDT and TriMM remained non-toxic. The degree of cytotoxicity correlated with the extent of metabolism for these analogues. The products of biotransformation of these compounds were stable precursors of formaldehyde (presumably *N*-hydroxymethyl intermediates) (FP) rather than formaldehyde itself. After injection of these 6 compounds to Balb/c mice the levels of FP generated in the plasma were markedly greater for the three active antitumour agents than for the inactive analogs. No free formaldehyde was detected in the plasma after administration of any of the compounds.

These results suggest that for these compounds *in vitro* cytotoxicity correlates with *in vitro* biotransformation and their antitumour activity correlates with plasma levels of FP generated by metabolism *in vivo*.

Certain *N*-methylmelamines, namely hexamethylmelamine (HMM) and pentamethylmelamine (PMM), have shown antitumour activity against murine and rat (for HMM) tumour models [2, 3] and have also been investigated in patients [4–8]. The mechanism of action of the *N*-methylmelamines is poorly understood but there is strong evidence to suggest that both metabolic activation and the presence of *N*-methyl groups are required if a melamine derivative is to exert significant antitumour activity [2, 9, 10].

N-Methylmelamines are metabolized by oxidative *N*-demethylation and this has been demonstrated both *in vitro* [2, 10] and *in vivo* [11–13]. This biotransformation proceeds via the generation of an *N*-hydroxymethyl (carbinolamine) intermediate which subsequently degrades to produce formaldehyde [14]. The carbinolamines of the *N*-methylmelamines are however relatively stable species [15] and *N*-hydroxymethylpentamethylmelamine (HMPMM) has been shown to be the major metabolite of HMM *in vitro* [16]. Both formaldehyde and *N*-hydroxymethylmelamine derivatives are directly cytotoxic to various murine and rat tumour cell lines *in vitro* although there is conflicting evidence as to the relative importance of these two species with respect to cytotoxicity [17–19]. Rutty and Connors have shown previously that the *N*-demethylation of *N*-methylmelamines *in vitro* to produce "formaldehyde equivalents" is predictive of the antitumour activity of these compounds against the murine ADJ/PC6A(PC6) plasmacytoma [2]. However the techniques used in this study did not distinguish between the generation of formaldehyde and its more stable precursors such as *N*-hydroxymethyl metabolites of *N*-methylmelamines. There are also some exceptions to this correlation, e.g. 2-chloro-4,6-bis(dimethylamino)-1,3,5-triazine (CBDT) which undergoes extensive oxidative *N*-demethylation *in vitro* and yet is devoid of antitumour activity *in vivo*.

It was the purpose of this study to examine the reasons for such exceptions and to clarify the respective roles of *N*-hydroxymethyl compounds and formaldehyde in the cytotoxic and antitumour activity of various *N*-methylmelamines. This was performed by

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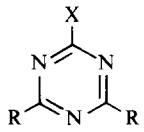

Fig. 1. Structures of analogues of hexamethylmelamine.

Compound	X	R
HMM	$N(CH_3)_2$	$N(CH_3)_2$
PMM	$NHCH_3$	$N(CH_3)_2$
TMM	NH_2	$N(CH_3)_2$
TriMM	$NHCH_3$	$NHCH_3$
CBDT	Cl	$N(CH_3)_2$
HBDT	$NHNH_2$	$N(CH_3)_2$

an investigation of the effect of biotransformation on the *in vitro* cytotoxicity of six *N*-methylmelamines which were either active or inactive against the PC6 plasmacytoma *in vivo*. The *in vitro* cytotoxicity and the *in vivo* antitumour activity of these compounds was then compared with the extent of their metabolism to formaldehyde or to its precursors, both *in vitro* and *in vivo*.

MATERIALS AND METHODS

Compounds and animals. HMM and its analogues (Fig. 1) were synthesized according to published methods [20, 21]. All other chemicals were commercially available.

Balb/c and BDF_1 mice (18–23 g) were obtained from Bantin and Kingman Ltd., Hull, U.K. Drugs, dissolved in either acetone:arachis oil (1:10), dimethylsulphoxide:arachis oil (1:10) or saline were administered by the i.p. route.

In vitro *N-demethylation*. Microsomes were prepared from the livers of male Balb/c mice according to the method of Schenkman and Cinti [22]. Reaction mixtures consisted of microsomal suspension (≡0.1 g wet liver weight), $MgCl_2$ (5 mM) and sufficient G6P, G6P dehydrogenase and NADP to generate NADPH (0.5 mM) in a final volume of Earl's buffer of 2.5 ml. Reactions were initiated by the addition of substrate (0.5 mM) in acetone (0.1 ml), incubated at 37° 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 were measured according to the method of Nash [23].

Maintenance of the PC6 *plasmacytoma*. The ascitic PC6 tumour cell line was passaged at 7 day intervals by i.p. injection of undiluted ascites (0.5 ml) into female Balb/c mice.

In vitro–in vivo *bioassay*. Estimates of the *in vitro* cytotoxicity of the analogues of HMM were obtained by the following method [24]. Ascites cells (2×10^6/ml) were incubated in RPMI 1640 medium:horse serum (6:4) in the presence of the drug (5 mM), liver microsomes (≡0.1 g wet liver weight/ml) and either with or without sufficient G6P, G6P dehydrogenase and NADP to generate NADPH (0.5 mM). Microsomes were prepared from the livers of male Balb/c mice as described [22] and incubations (2.5 ml) were performed for 2 hr in open beakers. Subsequently 10^6 cells (0.5 ml suspension) were injected i.p. into groups of 4 female Balb/c mice. After 7 days animals were killed by cervical dislocation and the ascites removed by lavage of the peritoneum using saline. Inspection of the peritoneal cavity revealed no solid tumour deposits. Cells were counted using a ZBI 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 which were counted in identical fashion. In this assay a direct relationship between the number of cells injected and the numbers harvested was observed.

Assay to determine between free formaldehyde and its precursors produced during N-*demethylation* in vitro. This was performed essentially as described in ref. [25]. In this assay substrates are incubated with hepatic microsomes for 30 min. At the end of the incubation period microsomes are precipitated by centrifugation and an aliquot of the supernatant is incubated with liver homogenate freed from microsomes. Metabolically generated formaldehyde is oxidised by the formaldehyde dehydrogenases which are abundant in mitochondria and liver cell cytosol but virtually absent in microsomes [26]. The method assumes that formaldehyde precursors are not metabolized by these enzymes and are detectable analytically as formaldehyde at the end of the incubation. This has been shown previously for HMPMM [25].

Substrates were used at a concentration of 0.5 mM except in the case of 2,2,4,4-tetramethylmelamine (TMM; 1 mM) and 2,4,6-trimethylmelamine (TriMM; 5 mM). 2,4-Bis(dimethylamino)-6-hydrazino-1,3,5-triazine (HBDT) could not be tested in this assay due to its poor *N*-demethylation at low concentrations (0.5 mM) and its insolubility at high concentrations (5 mM). Microsomal suspension (0.8 g wet liver weight) was added to each incubation and all other conditions have previously been described [25]. Control incubations were performed as described previously [27].

Antitumour assays

M5076 (M5) ovarian sarcoma. A suspension of 10^6 M5 tumour cells (from a routine passage grown as a solid s.c. tumour in BDF_1 mice) was injected i.m. into the left hind legs of groups of 5 or 10 female BDF_1 mice (18–23 g). Drugs were administered daily for 17 days i.e. approximately half the life span of the control tumour-bearing animals. Mean tumour volumes were measured by calipers every fourth day from day 12 until day 24 and the mean tumour volume index calculated by the standard method [28]. The ID_{90} refers to that dose which will produce 90% inhibition of the mean tumour volume of treated mice as compared to the mean tumour volume of control mice on day 24. LD_{10} and LD_{50} values were determined graphically using a range of doses varying from non-lethal to 100% mortality.

ADJ/PC6A (PC6) plasmacytoma. A suspension of 10^6 PC6 tumour cells (from a routine passage grown as a solid s.c. tumour in Balb/c mice) was injected

Table 1. Comparison of *in vitro* cytotoxicity with *in vitro* demethylation for selected analogues of HMM

Compound	*In vitro* cytotoxicity (% inhibition of PC6 tumour) −Activation	+Activation	*In vitro* demethylation*
HMM	0	94	100
PMM	0	>99	98
TMM	0	60	38
TriMM	0	3	6
CBDT	63	96	113
HBDT	24	11	3

* Relative to HMM.

i.m. into the left hind legs of groups of five female Balb/c mice (18–23 g). Drug treatment was initiated 14 days post implant at which point the tumours measured approximately 1–2 cm^3 in size. Drugs were administered daily from day 14 until day 18. Tumour volumes were measured by calipers on day 24 post implant and the mean tumour volume index calculated by the standard method [28]. A therapeutic index (T.I.) was calculated where T.I. = LD_{50}/ID_{90} (ID_{90} is defined as above).

Plasma levels of formaldehyde precursors in vivo. Drugs were injected i.p. into male Balb/c mice at a dose of 0.48 mmoles/kg. Control animals received vehicle only. Animals were anaesthetized using a mixture of halothane, nitrous oxide and oxygen. Blood samples were taken by cardiac puncture using syringes containing heparin (2500 units/ml; 0.05 ml) and plasma was obtained by centrifugation of the blood for 1 min in an Eppendorf 5412 centrifuge. Plasma samples were then assayed using a modification of the colorimetric method of Sawicki *et al.* [29].

Duplicate samples (0.25 ml) were mixed with 0.4% w/v 3-methyl-2-benzothiazoline hydrazone hydrochloride solution (0.25 ml). Samples were then left at either 0° or at 60° 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 and the reactions were terminated by the addition of acetone (3.2 ml). The optical density of the resultant solutions was then measured at 670 nm.

Calibration curves ($r > 0.99$) obtained using this method showed that formaldehyde could be determined in the presence of HMPMM at 0°, presumably because the *N*-methylol did not degrade to release formaldehyde under these conditions. At 60° however both formaldehyde and HMPMM were detected by this assay and the colorimetric response was shown to be additive. It cannot be excluded that formaldehyde precursors other than HMPMM were present and behaved like HMPMM under the conditions of this assay.

RESULTS

In vitro N-*demethylation*. The extent of *N*-demethylation of the analogues of HMM relative to HMM is shown in Table 1.

In vitro–in vivo *bioassays*. The cytotoxicity of HMM and its analogues against a PC6 cell line is shown in Table 1. Only CBDT proved to be significantly cytotoxic at a concentration of 5 mM in the absence of a mouse liver preparation. Only after biotransformation did the antitumour agents HMM, PMM and TMM prove to be cytotoxic while those agents which were inactive *in vivo* TriMM and HBDT, could not be metabolically activated. Interestingly CBDT, which was also inactive *in vivo* became more cytotoxic *in vitro* on metabolism. This increase in cytotoxicity in the presence of a metabolising system paralleled the ability of these agents to undergo *N*-demethylation *in vitro* (Table 1).

Assay to distinguish between free formaldehyde and its precursors produced during in vitro N-*demethylation*. The results, shown in Fig. 2, demonstrate that all of the compounds tested generate stable precursors of formaldehyde rather than free formaldehyde itself during the metabolic oxidation of the *N*-methyl moiety *in vitro*. The amount of Nash-positive species obtained with all of the compounds tested increased during incubation with microsome-free homogenate. The reason for this is unclear but presumably reflects the metabolism of a Nash-nega-

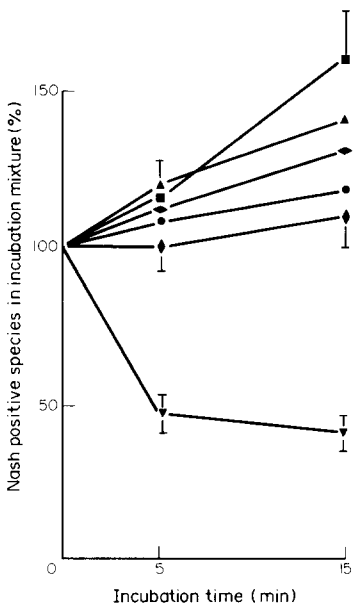

Fig. 2. Metabolism of Nash-positive microsomal metabolites of HMM (●), PMM (◆), TMM (■), TriMM (▲) and CBDT (◀▶) by mouse liver homogenate free from microsomes. ▼ indicates disappearance of formaldehyde. Values are the mean ± S.D. of at least 3 experiments.

Table 2. Comparison of *in vivo* antitumour activity with plasma levels of formaldehyde precursors for selected analogues of HMM

Compound	Antitumour activity M5076 sarcoma	Antitumour activity ADJ/PC6A plasmacytoma	Plasma levels of formaldehyde precursors *in vivo*‡ Peak level (nmoles/ml ± S.D.)	Plasma levels of formaldehyde precursors *in vivo*‡ AUC* (nmoles/ml × min ± S.D.)
HMM	+	+†	111 (±3)	14,580 (±3330)
PMM	+	+†	243 (±27)	21,300 (±6300)
TMM	+	+†	101 (±15)	7530 (±3121)
TriMM	−	−†	51 (±18)	3060 (±93)
CBDT	−	−†	16 (±26)	96 (±276)
HBDT	−	−	33 (±9)	3540 (±810)

* Area under curve (calculated by trapezoidal rule).
† Rutty and Connors [2].
‡ After i.p. administration of 0.48 mmoles/kg compound.

tive microsomal metabolite to a Nash-positive species by an enzyme system present in the post microsomal supernatant.

All of the above with the exception of HBDT have previously been tested by Rutty and Connors against the PC6 tumour [2] and their results are used in Table 2. HBDT when tested by our protocol proved to be completely inactive against this tumour even at a dose equalling the LD_{50} (LD_{10} = 147 mg/kg/day, LD_{50} = 208 mg/kg/day for 5 daily injections). Previous experiments have indicated that the tumour when implanted at the intramuscular site responds in an almost identical manner to the subcutaneous tumour [S.P. Langdon, unpublished data].

In vivo *plasma levels of formaldehyde precursors*. The levels of formaldehyde precursors generated in the plasma after the injection of 0.48 mmoles/kg i.p. of HMM and its analogues are shown in Fig. 3 and Table 2. Formaldehyde could not be detected in the plasma of mice after the administration of any of these compounds. It is clear from the results in Table 2 that those agents which are active antitumour agents (HMM, PMM and TMM) generated greater quantities of formaldehyde precursors (whether quantified on peak level or area under the plasma concentration versus time curve) in the plasma than their inactive analogues (TriMM, CBDT and HBDT).

DISCUSSION

The results of the *in vitro* *N*-demethylation assays using the Nash method to detect both formaldehyde and its precursors (Table 1) confirm the results of Rutty and Connors [2] who used rat rather than mouse liver as a source of microsomal *N*-demethylase activity. To determine whether these Nash positive metabolites were formaldehyde or its more stable precursors the products of microsomal metabolism were re-incubated with an enzyme source which removes free formaldehyde. The results (Fig. 2) indicate that the *in vitro* *N*-demethylation of the melamine derivatives tested lead to the production of stable precursors of formaldehyde rather than formaldehyde itself in each case.

The extent of the *in vitro* biotransformation of *N*-methylmelamines was paralleled by their *in vitro* cytotoxicity against a PC6 cell line when they were incubated with a hepatic metabolizing system. The presence of microsomes significantly increased the cytotoxicity of HMM, PMM and TMM and also, interestingly, CBDT even though this compound was directly cytotoxic. The two compounds which were poor substrates for microsomal *N*-demethylases—TriMM and HBDT—were not cytotoxic to PC6 cells in the presence of a microsomal system (Table 1). Thus these results suggest that the *in vitro* cytotoxicity of the *N*-methylmelamines tested can be

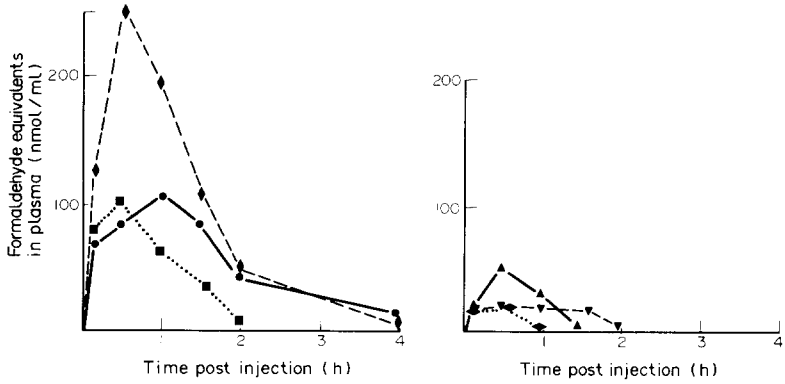

Fig. 3. *In vivo* plasma levels of formaldehyde precursors after i.p. administration of methylmelamines (0.48 mmol/kg) to Balb/c mice. ●—● HMM, ♦—♦ PMM, ■···■ TMM, ▲—▲ TriMM, ▼—▼ HBDT and ◆..◆ CBDT.

correlated with their *in vitro* *N*-demethylation. Rutty and Abel have shown previously that the *N*-desmethyl analogues of HMM become progressively less cytotoxic with the successive removal of *N*-methyl groups [19]. It therefore seems unlikely that the metabolic production of *N*-desmethyl derivatives of HMM is responsible for the increase in cytotoxicity to the PC6 cells observed after bioactivation. More likely candidates for the cytotoxic species produced during metabolism are formaldehyde and *N*-hydroxymethyl derivatives of *N*-methylmelamines. In this context it is pertinent to stress that formaldehyde precursors, rather than formaldehyde, were the major products of the *in vitro* *N*-demethylation of the *N*-methylmelamines (Fig. 2).

Rutty *et al.* have shown that formaldehyde equivalents were present in the plasma of mice which had received *N*-methylmelamines [13]. However these authors were uncertain whether these formaldehyde equivalents represented free formaldehyde or formaldehyde produced during the procedure of the Nash assay from the breakdown of a formaldehyde precursor. This was clarified by the use of a technique which distinguishes between formaldehyde and its precursors. The results confirm that the formaldehyde-like metabolites seen in the plasma of mice given *N*-methylmelamines were actually formaldehyde precursors rather than formaldehyde itself (Fig. 2). Rutty *et al.* did not observe a difference between the blood levels of formaldehyde equivalents produced from an injection of TriMM (90 mg/kg) and HMM (90 mg/kg). We found however that this was not the case when the compounds were injected at equimolar doses as can be seen in Fig. 3. The levels of formaldehyde precursors found in the plasma of mice after the injection of various *N*-methylmelamines differed and those compounds (HMM, PMM and TMM) which produced greater levels of precursors were also the very compounds which exhibited significant antitumour activity *in vivo* (Table 2). CBDT, like HBDT, produced only low levels of formaldehyde precursors in the plasma of mice and these two compounds were correspondingly inactive against both the PC6 and M5 tumours *in vivo*. The production of only low formaldehyde precursor levels following the injection of CBDT may reflect the labile nature of the chloro group [30]. Reaction with bionucleophiles may account for the direct (i.e. unactivated) cytoxicity of CBDT. It is also feasible that the chloro substituent may be replaced *in vivo* by endogenous nucleophiles such as glutathione, as shown for the melamine derivative atrazine [31], and the polarity of the resulting conjugate may preclude its subsequent *N*-demethylation.

These results demonstrate a correlation between the antitumour activity of the *N*-methylmelamines tested and the plasma levels of formaldehyde precursors they produce. However if this relationship was a quantitative correlation PMM, which produces significantly higher peak levels and greater AUC values or formaldehyde precursors than the other model compounds, would be expected to be a better antitumour agent than HMM. This is however not the case and both PMM and HMM have similar therapeutic indices against the PC6 [2] and M5 murine tumour models. The results however do suggest that there may be a plasma threshold level of formaldehyde precursors which has to be exceeded if an *N*-methylmelamine is to exert significant antitumour activity. This level, in the case of the male Balb/c mouse, would appear to be intermediate between the amounts of formaldehyde precursors produced following the administration of TriMM and HMM. These findings are in accord with the observation that the antitumour activity of PMM in various species differs dramatically just like the peak levels of formaldehyde precursors found in the plasma of these species after the injection of PMM [32]. The results also offer an explanation of why CBDT, which undergoes substantial *N*-demethylation *in vitro*, is not an effective antitumour agent *in vivo*.

We are aware that the technique employed in this investigation to differentiate between formaldehyde and its precursors do not unequivocally characterize these precursors. It is feasible that a hydroxymethyl group may have been transferred either intact or via formaldehyde from a hydroxymethylmelamine to another species e.g. cysteine or glutathione. However, it seems probable that these precursors represent mono- or multi-*N*-hydroxymethyl derivatives of *N*-methyl melamines. Certainly *in vitro* a hydroxymethylmelamine is derived from HMM [16]. It is important to stress therefore that the production of a specific formaldehyde precursor rather than the total levels of such precursors may determine the antitumour activity of a particular *N*-methylmelamine. In this context it is of interest that preliminary results in our laboratories have indicated the presence of 2,4-bis(hydroxymethyl)-2,4,6,6-tetramethylmelamine and 2,4,6-tris(hydroxymethyl)-2,4,6-trimethylmelamine in the plasma of mice given HMPMM.

In summary the results presented suggest that while the *in vitro* cytotoxicity of *N*-methylmelamines can be correlated with the extent of their *in vitro* *N*-demethylation, the plasma levels of formaldehyde precursors found after injection of these compounds is more indicative of their *in vivo* antitumour activity. Thus it is suggested that for a methylmelamine to be an antitumour agent it should not only undergo ready bioactivation *in vivo* but also the formaldehyde precursors generated must be bioavailable to the tumour.

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