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Effect of a hepatic activation system on the antiproliferative activity of hexamethylmelamine against human tumor cell lines

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Summary. *Incubation of hexamethylmelamine for 1 h with human tumor cell lines in culture did not inhibit colony formation at concentrations up to the limit of drug solubility (200 μg/ml). When 1-h incubations were carried out in the presence of a 9,000 g rat liver supernatant preparation and an NADPH-generating system, hexamethylmelamine markedly reduced colony formation. Cyclophosphamide inhibition of colony formation was also dependent on the presence of a 9,000 g supernatant preparation and an NADPH-generating system in incubation mixtures. A 1-h incubation of N-methylolpentamethylmelamine (a DNA-alkylating metabolite formed during N-demethylation of hexamethylmelamine) with human tumor cell lines reduced colony formation in the absence of the liver-activating system. Substantial NADPH-dependent N-demthylation of hexamethylmelamine was observed with rat liver, lung, and kidney microsomal preparations. In contrast, little or no HMM metabolism was observed with tumor cells, tumor cell homogenates, or NADPH-fortified tumor cell microsomal preparations. NADPH-dependent formation of cytotoxic metabolites is a prerequisite for antiproliferative activity of hexamethylmelamine against these human tumor cell lines. In vivo acitvity of hexamethylmelamine against some tumors may require metabolism by normal cells and subsequent transport of active species to the tumor site.*

Introduction

Hexamethylmelamine (HMM, Fig. 1) is an s-triazine antitumor agent which exhibits activity against a variety of human malignancies [8, 16]. HMM is extensively metabolized via *N*-demethylation by hepatic microsomal preparations, and in vivo following administration to mice, rats, and humans [1, 2, 4, 13, 19, 23, 24]. It has previously been suggested that metabolism is required for the antitumor activity of HMM [1, 13, 19, 20]. The detailed mechanism of action has not been described. While HMM and its demethylated metabolites are stable molecules, we have documented that HMM is activated by hepatic microsomal P-450 monooxygenases to reactive intermediates which covalently bind to microsomal protein [5, 6] and to calf thymus DNA [6]. Our results suggest that HMM antitumor activity is dependent on oxidative metabolic activation.

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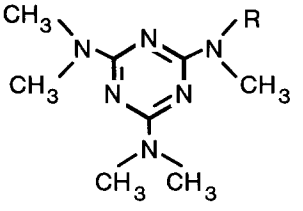

R = CH_3, HMM

R = CH_2OH, MPMM

Fig. 1. Structures of hexamethylmelamine (HMM) and *N*-methylolpentamethyl-melamine (MPMM)

To determine whether oxidative metabolism affects HMM cytotoxicity, cultured human tumor cell lines were incubated with HMM in the presence and absence of rat 9,000 *g* hepatic supernatant preparations (S-9). Rates of HMM oxidative metabolism were measured in NADPH-fortified microsomal preparations of rat liver, lung, and kidney as well as in human tumor cells, cell homogenates, and NADPH-fortified cell microsomal preparations to determine to what extent these tissues or tumor cells contribute to the production of HMM-alkylating metabolites.

Materials and methods

Reagents. HMM hydrochloride, pentamethylmelamine hydrochloride (PMM), and cyclophosphamide were supplied by the Drug Synthesis and Chemistry Branch, Divison of Cancer Treatment, National Cancer Institute, Bethesda, Md. The synthesis of *N*-methylolpentamethylmelamine (MPMM, Fig. 1) has been reported elsewhere [21]. Mitomycin C was obtained from Bristol Laboratories, Syracuse, NY. Glucose-6-phosphate, glucose-6-phosphate dehydrogenase, and NADP were purchased from Boehringer Mannheim Company, Indianapolis, Ind. Dulbecco's modified Eagle's medium, L-glutamine, Coomassie blue, trypsin/EDTA, penicillin, streptomycin, amphotericin B, fetal bovine serum, and calf serum were obtained from Grand Island Biological Company, Grand Island, NY.

Plating assay. Human rhabdomyosarcoma (A204), melanoma (A101D), renal cell carcinoma (A498), and alveolar cell lung carcinoma (A549) continuous cell lines, originally supplied by the Frederick Cancer Center courtesy of Dr George Todaro,

were provided by Dr Michael Lieber, Mayo Clinic. Each cell line was maintained in 75-cm^2 tissue culture flasks (Falcon Plastics, Beckton-Dickinson and Co., Cockeysville, Md) with standard culture medium: Dulbecco's modified Eagle's medium (DMEM) containing calf serum (10%), L-glutamine (0.29 mg/ml), penicillin (120 U/ml), streptomycin (120 μg/ml), and amphotericin B (0.30 μg/ml). Culture medium was replenished twice weekly and cells were passaged weekly using a brief digestion with trypsin/EDTA solution (1 : 250 trypsin and EDTA, 0.2 g/l). Cells in log phase growth were harvested for colony formation assays. Cells were routinely monitored and found to be free of mycoplasma.

The colony-forming assay was developed in our laboratory to facilitate assessment of drug cytotoxicity against cells in culture [15]. To each cell culture dish (60 mm, Falcon 3002) was added 2 ml standard culture medium (containing 10% fetal bovine serum) followed by 1 ml standard culture medium containing 500 tumor cells. Cell cultures were left undisturbed in the incubator (37° C, 5% CO_2/95% air, 100% relative humidity) for 48 h. Stock drug solutions were prepared by dissolving mitomycin C, cyclophosphamide, and HMM hydrochloride in sterile distilled water (deionized). MPMM was dissolved in DMSO and then diluted with deionized water to yield 10% DMSO solutions. The stock drug solutions (10× final concentration) were freshly prepared and added to replicate culture dishes (0.33 ml). After 1 h incubation (37° C, 5% CO_2/95% air, 100% relative humidity) the dishes were rinsed twice with prewarmed DMEM. After rinsing, 4 ml standard culture medium (except with 10% fetal bovine serum instead of calf serum) was added to each plate. The plates were incubated (37° C, 5% CO_2/95% air, 100% relative humidity) for 10 days. Medium was removed by aspiration and the dishes washed twice with 1–2 ml prewarmed saline. Colonies were fixed and stained with 3 ml Coomassie blue reagent (2.5 g Coomassie brillant blue G-250 in 454 ml methanol, 454 ml water, 92 ml glacial acetic acid) for approximately 15 min. The plates were rinsed with tap water and the colonies counted with the aid of a colony counter (American Optics). Results were expressed as the ratio of the number of colonies in the dish to the number of colonies in the control dish times 100. Each test was conducted in triplicate. Cloning efficiencies for control cells of the four cell lines were 29% (A204), 28% (A549), 10% (A498), and 14% (A101D).

S-9 Activating system. A modification [3] of the procedure originally described by Garner et al. [12] was used to prepare the 9,000 *g* supernatant. All operations were carried out at 4° C in glassware. Rats treated with phenobarbital (80 mg/kg, IP) for 3 successive days were sacrificed by decapitation 24 h after the last injection. Livers were removed and perfused with ice-cold 0.15 *M* KCl. Perfused livers were weighed and transferred to a beaker containing 3 ml 0.15 *M* KCl/g wet weight of liver. The tissue was minced with scissors, homogenized with a glass tissue homogenizer, and centrifuged for 20 min at 9,000 *g* to obtain the S-9 fraction. The S-9 activating mixture contained the following components per milliliter: $Na_2HPO_4 \cdot 7\,H_2O$ (100μmol), NADP (4 μmol), $MgCl_2$ (8 μmol), KCl (33 μmol), Glucose-6-phosphate (5 μmol), and 30 mg 9,000 *g* supernatant protein. This mixture was filtered sterile (0.45-μm Gelman Acrodisc filter) prior to use.

Colony formation assay with S-9 activating system. Log phase cells were added to dishes and allowed to attach to plastic for 48 h as described above for the plating assay. After 48 h, medium was removed by aspiration and 570 μl complete medium was added to each dish. The S-9 activating system mixture (1.23 ml) and stock drug solution at 10× final concentration (200 μl) were added to plates for 1 h incubation (37° C, 5% CO_2/95% air, 100% relative humidity). Cells were then rinsed twice with prewarmed DMEM, and 4 ml standard culture medium (containing 10% fetal bovine serum)was added. After 10 days in the incubator (37° C, 5% CO_2/95% air, 100% relative humidity) plates were rinsed with prewarmed saline, and colonies were stained with Coomassie blue and counted.

Metabolism studies. Male Sprague-Dawley rats (150–250 g, Mayo Clinic) were maintained on laboratory chow and water ad libitum. Microsomes from liver, lung, and kidney homogenates were prepared by differential centrifugation techniques as described by Ernster et al. [10]. Microsomes were prepared from human tumor cells by sonication of 2×10^{10} cells followed by homogenization and differential centrifugation. Protein was determined by the method of Lowry et al. [17]. Microsomal incubations (3 ml total volume) contained the following components present in the indicated final concentrations: NADP (0.5 m*M*), Glucose-6-phosphate (25 m*M*), glucose-6-phosphate dehydrogenase (0.5 U/ml), magnesium chloride (5 m*M*), microsomal protein (2.0 mg/ml, and substrate (0.25 m*M* added in 50 μl acetone). HMM and PMM concentrations were determined by a nitrogen-phosphorus gas chromatographic assay previously described [4].

Results

The antiproliferative effects of HMM, cyclophosphamide, and mitomycin C on colony formation by human rhabdomyosarcoma cell line (A204) are shown in Table 1. The maximum

Table 1. Effect of HMM, cyclophosphamide, and mitomycin C following short-term and continuous exposure on colony formation by human rhabdomyosarcoma cell line A204

		% Colony survival	
Drug	Concentration (μg/ml)	Continuous (10 days)	Short-term (1 h)
HMM	0	100	100
	10	96±1	80±3
	25	83±3	91±4
	50	70±2	89±3
	100	37±5	84±2
	150	29±2	85±5
	200	22±3	76±4
Cyclophosphamide	0	100	100
	10	100±0	93±6
	20	99±1	95±6
	50		94±5
	100	96±2	86±3
	500	30±5	87±5
Mitomycin C	0.0	100	
	0.0004	97±2	
	0.004	56±8	
	0.04	32±3	
	0.004		92±4
	0.04		79±3
	0.4		29±7
	4.0		0±0

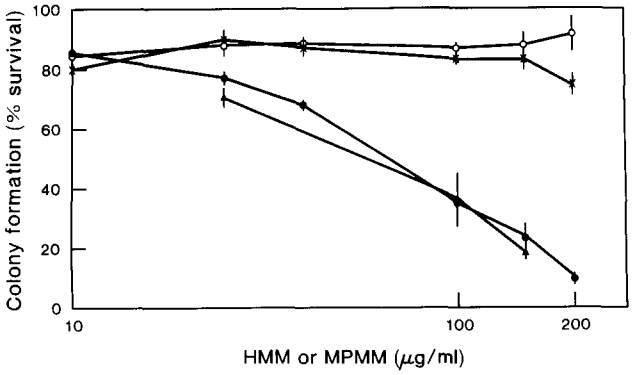

Fig. 2. Effect of HMM (○), HMM, and S-9 activating systems minus NADP (×), HMM and S-9 activating system plus NADP (●), and MPMM (▲) on colony formation by human rhabdomyosarcoma tumor cell lines (A204)

solubility of HMM in aqueous solution is approximately 200 μg/ml. HMM and cyclophosphamide were minimally cytotoxic following a 1-h exposure to cells at concentrations as high as 200 μg/ml and 500 μg/ml, respectively (Table 1). A 1-h exposure in mitomycin C reduced colony formation by 70% at concentrations as low as 0.4 μg/ml. Long-term (10-day) exposure of HMM and cyclophosphamide inhibited colony formation by 70% at concentrations of 150 μg/ml and 500 μg/ml, respectively. Following 10-day exposure to mitomycin C 68% was observed at a concentration of 0.04 μg/ml. Similar inhibition results were obtained with HMM, cyclophosphamide, and mitomycin C in melanoma (A101D), renal cell carcinoma (A498), and alveolar cell lung carcinoma (A549) cell lines (data not shown).

HMM and cyclophosphamide inhibited colony formation when incubated with A204 cells for 1 h in the presence of the hepatic metabolizing system (S-9). Colony inhibition was completely dependent upon the presence of the NADPH-generating system (Figs. 2 and 3). Colony formation was stimulated slightly by the S-9 mixture alone (data not shown). Results of 1-h incubation experiments with HMM and cyclophosphamide in all four cell lines are summarized in Table 2.

N-Methylolpentamethylmelamine (MPMM, Fig. 1), the intermediate formed during microsomal HMM *N*-demethylation, covalently binds to calf thymus DNA when HMM is incubated with hepatic microsomal preparations [6]. When MPMM was exposed to A204 cells in the absence of the S-9 activating system for 1 h at a concentration of 100 μg/ml, colony formation was inhibited by 69%, a similar result to those obtained with HMM in the presence of NADPH and the S-9 system (Fig. 2).

The extent to which normal tissues and tumor cells might contribute to the production of the cytotoxic intermediate MPMM was assessed by determining NADPH-dependent HMM demethylation in rat liver, lung, and kidney preparations and human tumor cells and subcellular preparations. Substantial NADPH-dependent *N*-demethylation of HMM was observed in rat liver, lung, and kidney microsomal preparations, while very little NADPH-dependent *N*-demethylation of HMM was observed with tumor cell microsomal preparations (Fig. 4). No HMM *N*-demethylation was detected with whole tumor cells (1-h or 10-day incubations) or with tumor cell homogenates.

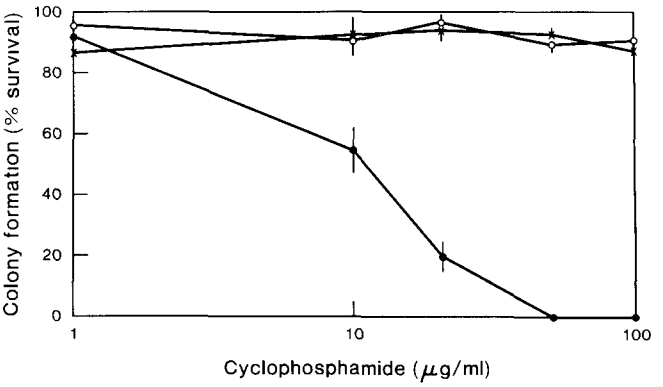

Fig. 3. Effects of cyclophosphamide (○), cyclophosphamide and S-9 activating system minus NADP (×), and cyclophosphamide and S-9 activating system plus NADP (●) on colony formation by human rhabdomyosarcoma cell line (A204)

Table 2. Effect of HMM and cyclophosphamide in the presence of S-9 activation system following short-term (1-h) exposure on colony formation by continuous human tumor cell lines (± NADP cofactor)

		% Colony survival (± NADP)							
Drug	Concentration (μg/ml)	A204		A549		A498		A101D	
		+	−	+	−	+	−	+	−
HMM	0	100	100	100	100	100	100	100	100
	25	76±3	89±4	94±0	96±0	90±9	100±9	96± 0	93±0
	40	68±2	91±2	88±1	95±6	65±9	85±6	92±17	97±4
	100	30±2	88±3	79±0	103±0	33±9	84±5	120± 4	98±4
	150	19±6	91±5	64±7	99±3	27±1	95±2	92± 0	101±0
	200	11±2	94±7	69±0	88±0	9±0	81±0	71± 0	92±0
Cyclophosphamide	0	100	100	100	100	100	100	100	100
	1	92±5	96±1	101±0	103±0	92±8	102±6	55±12	96±5
	10	55±9	93±2	104±0	104±0	108±0	105±0	52±14	89±7
	20	21±6	97±3	86±1	98±1	79±2	91±4	26±13	92±3
	100	21±10	87±3	81±0	99±0	9±5	96±6	0	93±2
	500	0	97±4	3±0	102±0	0	86±0	0	101±0

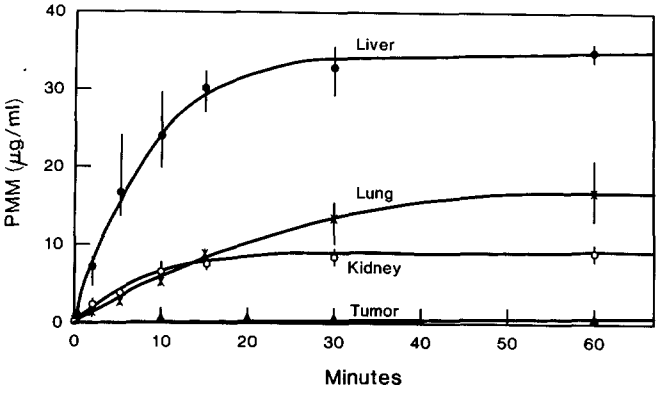

Fig. 4. Appearance of HMM metabolite, PMM, on incubations of NADPH-fortified microsomal preparations of rat liver (●), lung (×), kidney (○) and human tumor lines (▲) at 37° C

Discussion

The antiproliferative activity of HMM against human tumor cell lines is dependent on the presence of NADPH and a hepatic metabolizing system. Incubation of the HMM metabolite, MPMM, with human tumor cells for 1 h inhibits colony formation to the same extent as HMM, but without addition of the metabolizing system or NADPH. We previously reported that liver NADPH-dependent cytochrome P-450 monooxygenases convert radiolabeled HMM to reactive metabolites which covalently bind to calf thymus DNA [5, 6]. Based on the results of those studies, we suggested that MPMM, the carbinolamine intermediate formed during cytochrome P-450-mediated HMM *N*-demethylation [13], was the major reactive species binding to DNA [6]. Covalent binding was observed when radiolabeled MPMM [21] was incubated with DNA in the absence of microsomal preparations [6]. These results are consistent with the hypothesis that HMM cytotoxicity against cells in culture is dependent on NADPH-dependent cytochrome P-450 conversion of HMM to the DNA-alkylating metabolite MPMM. Demethylated HMM metabolites, also formed via intermediate carbinolamines, are less likely to play a major role in HMM cytotoxicity. Pentamethylmelamine, the initial HMM demethylation product, is converted to alkylating species by microsomal preparations to a much lesser extent than is HMM [5]. Formaldehyde (a weak alkylating agent) generated during cytochrome P-450-mediated demethylation of HMM binds primarily to protein rather than DNA, and thus is not likely to be involved in HMM cytotoxicity [4].

There are at least two reports of cell lines which metabolize HMM, a murine ovarian cell line [11] and L15784 lymphoblasts [7]. HMM is active against the murine ovarian cell line [22]. In contrast, Rutty and Abel have suggested that results of studies with the Walker ascites tumor are best explained by proposing that HMM metabolism by normal tissue is required for HMM activity in this system [18]. HMM is active against the tumor in vivo, but inactive against Walker ascites cells in culture. MPMM, however, is active against the Walker line in vitro [18]. Neither HMM metabolism by tumor cells nor the effect of a liver S-9 system on in vitro cytotoxicity of HMM was determined in these studies.

The human tumor cell lines used in our studies did not metabolize HMM. Even concentrated NADPH-fortified microsomal preparations of tumor cells only minimally demethylated HMM. In contrast, microsomal preparations from several normal tissues readily demethylated HMM. MPMM formed during HMM demethylation [13] has a half-life in buffer at physiological pH, or in a mixture of buffer and microsomal protein, of 30–60 min [6, 13]. Thus, MPMM could act as a transport form of the ultimate alkylating species (probably an iminium ion species) [6], just as the 4-hydroxy metabolite of cyclophosphamide has been suggested to be a transport form of the ultimate alkylating metabolites [14]. The precise relationship between HMM metabolism (by tumor cells and/or normal cells) and in vivo HMM antitumor activity remains to be established. Based on results of our metabolic activation studies with HMM, we are currently preparing several analogs which will alkylate DNA without metabolism of the parent molecule. These molecules should not be dependent on metabolism by normal tissues or tumor cells for in vivo antitumor activity.

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