

Toxicity and Antitumor Activity of Hexamethylmelamine and Its *N*-Demethylated Metabolites in Mice with Transplantable Tumors¹

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SUMMARY

N-Demethylated metabolites of the antineoplastic agent hexamethylmelamine were synthesized, and their toxicities and antitumor activities were determined *in vivo*. Determinations of the lethal dose for 10% of the male C57BL × DBA/2 F₁ (hereafter called BD2F₁) mice showed hexamethylmelamine toxicity to be decreased by *N*-demethylation; the metabolites showed a direct relationship between potency (mmoles/kg/day) and number of methyl groups present. In BD2F₁ mice bearing Sarcoma 180 or Lewis lung carcinoma, the antitumor activities of the methylmelamines decreased with a reduction in number of methyl groups, but were similar at equitoxic levels. Results were similar in L1210 leukemic mice treated with lethal dose levels of the metabolites for 10% of the mice when mean survival times were measured. The therapeutic equality produced with equitoxic levels, together with the ineffectiveness of melamine, suggested that the presence of a methyl group, rather than the number, was the determining factor in the antitumor activity of the methylmelamines.

INTRODUCTION

Completed Phase I and Phase II trials (22-25) showed HMM³ to be a clinically effective antitumor agent. A report by Blum *et al.* (1) listed small-cell (oat) carcinoma of the lung, ovarian adenocarcinoma, lymphoma, and breast cancer as the tumor types that responded to HMM. The mechanism by which the drug exerts its antitumor action remains unknown. Toxicity and clinical response rates (1), as well as close structural similarity to the known alkylator, triethylenemelamine, suggested an alkylating action for HMM. Worzalla *et al.* (26), as part of a study to identify the metabolic pathways of HMM, tested the drug and its *N*-demethylated metabolites for alkylating activity using the chemical indicator 4-(*p*-nitrobenzyl)pyridine. None of the compounds exhibited alkylating activity. Negative results were also obtained when the compounds were assayed

in vitro for their ability to inhibit dihydrofolate reductase (27). These results duplicated the conclusions of Bořkovec and DeMilo (2). They studied the chemosterilizing activity of HMM in houseflies and concluded that HMM was neither an alkylating agent nor a folate antagonist.

Worzalla *et al.* (27) suggested that the metabolism of HMM might result in an *N*-demethylated homolog that would be more active and/or less toxic than the parent compound. The present study was designed to test this hypothesis by examining the compounds in tumor systems in which HMM was reported to be inactive (L1210 leukemia) and moderately active (Sarcoma 180) (8). A 3rd tumor system (Lewis lung carcinoma) was also used, since it has been included in the drug screening program of the NCI and Sarcoma 180 has been dropped. Data from the study were analyzed for structural relationships indicating antitumor activity and a possible mode of action.

MATERIALS AND METHODS

Chemicals. HMM was provided by the Clinical Branch, Collaborative Research, NCI, USPHS, and was used as received. Melamine (Aldrich Chemical Co., Milwaukee, Wis.) was recrystallized from water. Pentamethylmelamine, *N*²,*N*²,*N*⁴,*N*⁴-tetramethylmelamine, *N*²,*N*²,*N*⁴,*N*⁶-tetramethylmelamine, *N*²,*N*²,*N*⁴-trimethylmelamine, *N*²,*N*⁴,*N*⁶-trimethylmelamine, *N*²,*N*²-dimethylmelamine, and *N*²,*N*⁴-dimethylmelamine were synthesized using the methods described by Bořkovec and DeMilo (2), and monomethylmelamine was synthesized according to the method of Pearlman and Banks (13). Melting points were identical to literature values and purity was greater than 99% by gas chromatography.

Injection solutions were prepared for each dose level by adding the weighed drug to 0.05 N HCl (two-thirds of final volume), titrating with 1.0 N HCl until dissolution, and diluting to final volume with 0.05 N HCl. Final pH of solutions was about 1.45. Concentrations were adjusted so that 0.01 ml/g body weight was administered *i.p.* Melamine, monomethylmelamine, and *N*²,*N*²-dimethylmelamine were prepared as suspensions in 1% sodium carboxymethylcellulose.

Animals. Male BD2F₁ mice (A. R. Schmidt, Madison, Wis.) weighing 18 to 20 g were housed for at least 1 week for acclimatization before use and were provided Purina laboratory chow and tap water *ad libitum* for the duration of the experiment.

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³The abbreviations used are: HMM, hexamethylmelamine [2,4,6-tris(dimethylamino)-s-triazine] (NSC-13875); NCI, National Cancer Institute; LD₁₀, lethal dose for 10% of the mice; MED, minimum effective dose.

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Tumor Lines. Sarcoma 180 was maintained by serial s.c. transplantation into BD2F₁ mice every 7 days. Lewis lung carcinoma (generously provided by Dr. Bernard Kline, Wisconsin Alumni Research Foundation Institute, Madison, Wis.) was maintained by serial s.c. transplantation into C57BL/6 mice every 14 days. Tumor tissue was removed aseptically into Eagle's medium (Minimum Essential Medium, Microbiological Associates, Inc., Bethesda, Md.), trimmed of necrotic and fibrous tissue, and minced with scissors into fragments for s.c. implantation into the flank region using a 16-gauge trocar. Tumor fragments were tested for sterility by placing samples into tubes containing thioglycolate broth (Difco Laboratories, Detroit, Mich.) and checking for bacterial growth after 48 hr. If growth was found, the experiment was discarded.

L1210 leukemia (obtained from Dr. Kline) was maintained by weekly passage of 10⁵ cells i.p. in DBA/2 mice (A. R. Schmidt Co., Madison, Wis.). For determination of antitumor activity, male BD2F₁ mice were inoculated i.p. with 10⁵ ascites cells.

Toxicity. Acclimatized BD2F₁ mice were randomized into groups of 6 animals/dose level, weighed, given i.p. injections once daily for 5 consecutive days, weighed on Day 5, and observed through Day 12 for deaths. To determine LD₁₀ values, a line of best fit was drawn for each compound using linear regression analysis on the results, expressed as log dose *versus* probit of percentage survivors. A minimum of 4 doses was used for evaluation of each compound.

Therapeutic Studies. Groups of 10 BD2F₁ mice/dosage regimen were given tumor transplants on Day 0, i.p. injections of 0.01 ml/g body weight of drug solution on Days 1 to 5, and sacrificed on Day 12. Tumors were excised and weighed (± 10 mg) individually. Control mice (20/group) received 0.05 N HCl. Only those groups with 70% survival were used for evaluation. Tumors weighing ≤ 40 mg in control mice were labeled "no takes." Designation of "cure" or no take in the treated groups was based on NCI Drug Research and Development Division standards (7). Percentage of tumor growth inhibition relative to control for each dose was analyzed using a linear regression technique on the log dose *versus* probit of percentage of tumor inhibition relationship. Four to 8 points in the dosage range of 0.5 to 1.0 \times LD₁₀ were used for each regression analysis. The dose required to produce 90% tumor growth inhibition, *i.e.*, MED, and the percentage of tumor growth inhibition at the LD₁₀ dose level were extrapolated from the linear regression line.

Increase in life-span studies using L1210 leukemia and Lewis lung carcinoma were done using the same 5-day injection schedule as above. Percentage of increase in life-span for L1210 was calculated from mean survival times (animals were checked for deaths every 6 to 12 hr). Median survival time was used for evaluating the Lewis lung data (animals were checked for deaths daily).

RESULTS

Toxicity of HMM and Its *N*-Demethylated Homologs. Table 1 shows that a decrease in potency occurred as the

number of methyl groups was reduced. The LD₁₀ values ranged from 0.46 mmoles/kg/day for HMM to 6.04 mmoles/kg/day for melamine, a spectrum characterized by a 13-fold decrease in potency. The decrease was gradual initially; the successive reductions in number of methyl groups from 6 to 3 were accompanied by small changes in the relative potency. When the number of methyl groups was reduced from 3 to 2, a precipitous drop in potency occurred. HMM was about 7 times more potent than were the 2 dimethylmelamines. Succeeding reductions in the remaining number of methyl groups gave homologs with lower relative potencies.

Toxicity of the compounds at their LD₁₀ levels is indicated by the percentage of weight loss produced with each compound for the 5-day injection period. As expected, the weight losses follow closely the relative potencies of the homologs; HMM and pentamethylmelamine produced the largest percentage weight loss, while monomethylmelamine and melamine produced the least. The low percentage weight loss induced with each of the tetramethylmelamines relative to that induced with the other homologs containing a comparable number of methyl groups could indicate that their extrapolated LD₁₀ values were lower than the actual values.

Effect of HMM and Its *N*-Demethylated Homologs on the Growth of Sarcoma 180 and Lewis Lung Tumors *in Vivo*. BD2F₁ mice (10/dose level) were given s.c. implants of either Sarcoma 180 or Lewis lung tumor fragments on Day 0. For each compound, a series of doses was administered i.p. once daily on Days 1 through 5. The mice were sacrificed on Day 12 and the tumors were removed. The average tumor weights of the treated mice were compared to the average tumor weight of the control mice to determine percentage of inhibition of tumor growth. A plot of the results as a log dose *versus* probit of percentage of inhibition relationship enabled the MED required to produce 90% inhibition of tumor growth, and the percentage of inhibition of tumor growth produced by the maximum tolerated dose, *i.e.*, LD₁₀, to be determined from the graph of each compound. Results are summarized in Tables 2 and 3.

The therapeutic potency (MED) of HMM relative to that of each of its *N*-demethylated homologs was almost quantitatively identical in each of the 2 tumor systems. Furthermore, the relative therapeutic potencies followed qualitatively the relative toxic potencies shown in Table 1. For example, as in the toxicity study, the loss of a methyl group from the trimethylmelamines resulted in the largest decrease in therapeutic potency; HMM was 11 times more potent than *N*²,*N*⁴-dimethylmelamine and 7 times more potent than *N*²,*N*²-dimethylmelamine. These numbers represent an average decrease of more than 200% in the MED (mmoles/kg/day) from that of the trimethylmelamines. It was significant that the absence of methyl groups, *i.e.*, melamine, resulted in an inactive compound.

There was an absence of a distinct pattern of diminishing efficacy (percentage of inhibition at the LD₁₀ level) concurrent with a reduction in the number of methyl groups. HMM and pentamethylmelamine exhibited the greatest efficacy. The lower activity of the tetramethylmelamines at their LD₁₀ levels would be expected if the extrapolated LD₁₀

Table 1

LD₁₀ values for HMM and its N-demethylated homologs

Groups of 6 BD2F₁ mice (at least 6 levels/drug) were given i.p. injections once daily for 5 days and weighed on Days 1 and 5, and survival was recorded through Day 12. LD₁₀ values were obtained using linear regression analysis on the log dose *versus* probit of percentage of survivors relationship.

Drug	LD ₁₀		Relative potency ^a	% wt loss ^b
	mg/kg/day	mmoles/kg/day		
HMM	96	0.46	1.0	14.4
Pentamethylmelamine	108	0.55	0.84	14.4
<i>N</i> ² , <i>N</i> ² , <i>N</i> ⁴ , <i>N</i> ⁴ -Tetramethylmelamine	78	0.43	1.07	7.3
<i>N</i> ² , <i>N</i> ² , <i>N</i> ⁴ , <i>N</i> ⁶ -Tetramethylmelamine	101	0.55	0.84	9.6
<i>N</i> ² , <i>N</i> ² , <i>N</i> ⁴ -Trimethylmelamine	155	0.92	0.50	11.0
<i>N</i> ² , <i>N</i> ⁴ , <i>N</i> ⁶ -Trimethylmelamine	207	1.23	0.37	12.5
<i>N</i> ² , <i>N</i> ² -Dimethylmelamine	465 ^c	3.02	0.15	7.8
<i>N</i> ² , <i>N</i> ⁴ -Dimethylmelamine	550	3.57	0.13	9.8
Monomethylmelamine	565 ^c	4.03	0.11	3.6
Melamine	762 ^c	6.04	0.08	1.0

^a Relative potency = LD₁₀ [HMM]/LD₁₀ [homolog] based on mmoles/kg/day.

^b Average percentage of weight loss on Day 5. Controls lost 3.5% receiving 0.01 ml per g body weight of 0.05 N HCl per day.

^c Drugs were given as suspensions in 1% sodium carboxymethylcellulose; all other drugs were dissolved in 0.05 N HCl.

Table 2

Therapeutic evaluation of HMM and its N-demethylated homologs in BD2F₁ mice bearing Sarcoma 180 tumors

Groups of 10 BD2F₁ mice were given s.c. implants of Sarcoma 180 tumor fragments via trocar on Day 0, treated i.p. once daily on Days 1 to 5 with drug levels ranging from 0.5 × LD₁₀ to LD₁₀, and sacrificed on Day 12, and tumors were excised and weighed. Control animals (20/group) received 0.01 ml of 0.05 N HCl per g of body weight. Percentage of inhibition of tumor growth was calculated using average tumor weight for each test group and control. Linear regression analysis performed on a log dose *versus* probit of percentage of inhibition relationship gave values for MED and percentage of inhibition at LD₁₀ for each drug.

Drug	MED ^a		Relative potency ^b	Therapeutic index ^c	% inhibition at LD ₁₀
	mg/kg/day	mmoles/kg/day			
HMM (80) ^d	108	0.51	1.0	0.89	82
Pentamethylmelamine (40)	155	0.79	0.65	0.70	75
<i>N</i> ² , <i>N</i> ² , <i>N</i> ⁴ , <i>N</i> ⁴ -Tetramethylmelamine (50)	148	0.81	0.63	0.53	49
<i>N</i> ² , <i>N</i> ² , <i>N</i> ⁴ , <i>N</i> ⁶ -Tetramethylmelamine (50)	170	0.93	0.55	0.59	49
<i>N</i> ² , <i>N</i> ² , <i>N</i> ⁴ -Trimethylmelamine (40)	195	1.16	0.44	0.79	62
<i>N</i> ² , <i>N</i> ⁴ , <i>N</i> ⁶ -Trimethylmelamine (80)	240	1.43	0.36	0.83	80
<i>N</i> ² , <i>N</i> ² -Dimethylmelamine (40)	640	4.15	0.12	0.73	61
<i>N</i> ² , <i>N</i> ⁴ -Dimethylmelamine (40)	980	6.36	0.08	0.56	33
Monomethylmelamine (40)	950	6.78	0.08	0.59	40
Melamine (20)	(Inactive)				

^a Dose that would produce a 90% inhibition of tumor growth.

^b Ratio of MED of HMM to MED of homolog (mmole/kg/day).

^c Ratio of LD₁₀ to MED (mg/kg/day) for each compound.

^d Numbers in parentheses, total number of mice used for therapeutic evaluation.

values were lower than the actual values suggested above. Still, the efficacies of the tetramethylmelamines, the trimethylmelamines, and *N*²,*N*²-dimethylmelamine were similar in magnitude. The low efficacy of *N*²,*N*⁴-dimethylmelamine contrasted with that of its positional isomer and was closer to that of monomethylmelamine. The range of efficacy produced with the dimethylmelamines encompassed the point at which a compound was characterized as active or inactive by the screening protocol of NCI, namely,

≥ 58% inhibition of tumor weight relative to the untreated controls (7). This suggested that the presence of 2 methyl groups was pivotal in determining whether a homolog was an effective antitumor compound.

The therapeutic index (LD₁₀/MED) for each of the methylmelamines was determined in order to find what effect *N*-demethylation had on the margin of safety of HMM. Tables 2 and 3 show no substantial differences in the therapeutic indices. None of the compounds had an index of

Table 3

Therapeutic evaluation of HMM and its *N*-demethylated homologs in BD2F₁ mice bearing Lewis lung carcinomas

Groups of 10 BD2F₁ mice were given s.c. implants of Lewis lung tumor fragments via trochar on Day 0, treated i.p. once daily on Days 1 to 5 with drug levels ranging from 0.5 × LD₁₀ to LD₁₀, and sacrificed on Day 12, and tumors were excised and weighed. Control animals (20/group) received 0.01 ml of 0.05 N HCl per g of body weight. Percentage of inhibition of tumor growth was calculated using average tumor weight for each test group and control. Linear regression analysis performed on a log dose *versus* probit of percentage of inhibition relationship gave values for MED and percentage of inhibition at LD₁₀ for each drug.

Drug	MED ^a		Relative potency ^b	Therapeutic index ^c	% inhibition at LD ₁₀
	mg/kg/day	mmoles/kg/day			
HMM (70) ^d	103	0.49	1.0	0.93	80
Pentamethylmelamine (70)	110	0.56	0.88	0.98	88
<i>N</i> ² , <i>N</i> ² , <i>N</i> ⁴ , <i>N</i> ⁴ -Tetramethylmelamine (40)	130	0.71	0.69	0.60	55
<i>N</i> ² , <i>N</i> ² , <i>N</i> ⁴ , <i>N</i> ⁶ -Tetramethylmelamine (50)	160	0.88	0.56	0.63	66
<i>N</i> ² , <i>N</i> ² , <i>N</i> ⁴ -Trimethylmelamine (50)	210	1.25	0.39	0.74	56
<i>N</i> ² , <i>N</i> ⁴ , <i>N</i> ⁶ -Trimethylmelamine (50)	238	1.42	0.35	0.87	74
<i>N</i> ² , <i>N</i> ² -Dimethylmelamine (40)	560	3.63	0.13	0.83	67
<i>N</i> ² , <i>N</i> ⁴ -Dimethylmelamine (40)	840	5.45	0.09	0.65	40
Monomethylmelamine (40)	1080	7.71	0.06	0.52	48
Melamine (20)	(Inactive)				

^a Dose that would produce a 90% inhibition of tumor growth.

^b Ratio of MED of HMM to MED of homolog (mmole/kg/day).

^c Ratio of LD₁₀ to MED (mg/kg/day) for each compound.

^d Numbers in parentheses, total number of mice used for therapeutic evaluation.

1.0 or greater, reflecting the undesirable overlap of toxicity at the dose necessary for effective inhibition of tumor growth.

Effect of HMM and Its *N*-Demethylated Homologs on Survival Times of BD2F₁ Mice Bearing Lewis Lung Tumors or L1210 Leukemia. The median survival times of BD2F₁ mice bearing Lewis lung tumors were measured as an additional index of the antitumor activity of each of the compounds for 2 reasons: (a) the parameter is an effective index of the activity of a drug because toxicity-related effects have been reported to have less influence on survival time than on tumor weight inhibition (15); and (b) the effectiveness of a compound on tumor weight inhibition does not indicate a similar degree of effectiveness in increasing the survival time of the tumor-bearing animals (5).

Two-thirds of the LD₁₀ level of each compound was administered using the injection schedule described in the tumor weight inhibition studies above. There was essentially no increase in the median survival of the treated groups over control groups (average increase was 4.4%, with a range of 0 to 12.1% increase in median survival time). The percentage of weight loss seen at two-thirds of the LD₁₀ (average weight loss was 7.7%) was not appreciably lower than that seen in Table 1 with the LD₁₀ level of each of the compounds. This suggests that toxicity, as measured by weight loss, may not be an accurate indicator of the antitumor effectiveness of these compounds.

For the studies involving L1210 leukemia, each of the compounds was administered at its LD₁₀ level using the 5-day injection schedule described previously. The results in Table 4 show that none of the compounds increased the mean survival times of the treated groups by 25% or greater;

thus, none was active as described by NCI's standard (7). The failure of melamine to significantly increase the mean survival time ($p > 0.05$) of the treated group beyond that of the control group was consistent with its demonstrated inactivity against the Sarcoma 180 (Table 2) and Lewis lung (Table 3) tumors. With the exceptions of *N*²,*N*²-dimethylmelamine and *N*²,*N*⁴,*N*⁶-trimethylmelamine, the presence of a methyl group(s) on the melamine nucleus resulted in metabolites that produced both significant ($p < 0.05$) and similar increases in the mean survival times of the respective groups beyond that of the control groups. The *N*²,*N*⁴,*N*⁶-trimethylmelamine-treated group had a mean survival time that was less than that of the control group. This, together with the largest percentage of weight loss of the treated groups, suggested an insidious toxicity not reflected in the number of toxic deaths produced. The large number of toxic deaths with *N*²,*N*²,*N*⁴,*N*⁴-tetramethylmelamine and *N*²,*N*⁴-dimethylmelamine was unexpected and may have been due to the low pH of the drug solutions (these compounds required more 1.0 N HCl to be put into solution, as explained under "Materials and Methods"). In view of the toxicity exhibited by *N*²,*N*⁴-dimethylmelamine, the gain in weight of that group was an anomaly that contrasted with the weight losses shown for the other groups.

DISCUSSION

Prior to this study, a number of compounds, later identified by Worzalla *et al.* (26) as metabolites of HMM, specifically melamine (6, 10, 17), *N*²,*N*²-dimethylmelamine (11, 19), *N*²,*N*⁴,*N*⁶-trimethylmelamine (14, 18), and *N*²,*N*²,*N*⁴,*N*⁶-tetramethylmelamine (20), were included in

Table 4
Effect of HMM and its *N*-demethylated homologs on survival times of BD2F₁ mice bearing L1210 leukemia
Drugs given i.p. once daily on Days 1 to 5 at LD₁₀ dose.

Experiment	Drug	Mean survival time (hr ± S.D.)	<i>p</i> ^a	% increase of mean survival time	% wt loss ^b	No. of toxic deaths ^c
A	Control ^d	168.6 ± 15.8			+2.6	0
	HMM	201.6 ± 25.2	<0.001	19.6	16.7	0
	Pentamethylmelamine	198.7 ± 26.9	<0.001	17.9	12.5	1
	<i>N</i> ² , <i>N</i> ² , <i>N</i> ⁴ , <i>N</i> ⁴ -Tetramethylmelamine	189.6 ± 5.4	<0.01	12.5	11.7	5
	<i>N</i> ² , <i>N</i> ² , <i>N</i> ⁴ , <i>N</i> ⁶ -Tetramethylmelamine	190.7 ± 4.0	<0.001	13.1	8.6	1
	<i>N</i> ² , <i>N</i> ² , <i>N</i> ⁴ -Trimethylmelamine	189.6 ± 30.4	<0.05	12.5	5.1	0
	<i>N</i> ² , <i>N</i> ⁴ , <i>N</i> ⁶ -Trimethylmelamine	158.7 ± 22.3	>0.05	0	21.2	1
	<i>N</i> ² , <i>N</i> ⁴ -Dimethylmelamine	196.0 ± 16.4	<0.01	16.3	+0.4	4
B	Control ^e	184.0 ± 14.8			10.5	0
	<i>N</i> ² , <i>N</i> ² -Dimethylmelamine	197.1 ± 28.4	>0.05	7.1	14.2	3
	Monomethylmelamine	202.5 ± 23.5	<0.05	10.1	12.0	2
	Melamine	190.5 ± 31.7	>0.05	3.5	10.2	2

^a Significance value on Student's *t* test. Mean survival time compared to control.

^b Percentage of weight loss produced in mice from Day 1 to Day 5.

^c Mice dead before Day 6. Toxic mice were not included in determining mean survival times.

^d Control mice received 0.01 ml of 0.05 N HCl per g body weight. All drugs in Experiment A were dissolved in 0.05 N HCl.

^e Control mice received 0.01 ml of 1% sodium carboxymethylcellulose per g body weight. All drugs in Experiment B were suspended in 1% sodium carboxymethylcellulose.

animal screening studies and reported to be inactive. These previous studies were carried out by several investigators at different times under varied conditions, making it impossible to evaluate structure-activity relationships. For this reason, all 9 methylmelamines and melamine were tested and evaluated as a group in this study.

The LD₁₀ values reported in Table 1 should be approximate to the actual values. The use of a minimum number of mice for each compound and for each dose level enhanced the probability that the extrapolated values were either above or below the actual values. The LD₁₀ values for the tetramethylmelamines, especially *N*²,*N*²,*N*⁴,*N*⁴-tetramethylmelamine, may be low, as shown by the smaller percentage of weight loss incurred over the 5-day injection period relative to that produced by the homologs with the next higher (pentamethylmelamine) and lower (trimethylmelamines) numbers of methyl groups (Table 1). Since the determination of efficacy, *i.e.*, the maximum percentage of tumor growth inhibition, was based on the extrapolated LD₁₀ values, any error in the LD₁₀ of a homolog would be transposed into the magnitude of the maximum percentage of tumor inhibition. Therefore, a stringent quantitative structure-activity analysis with respect to efficacy was not stressed, but instead, a qualitative analysis that described the pattern of activity (efficacy) that accompanied the *N*-demethylation of HMM was considered more realistic and accurate.

We demonstrated in these studies that *N*-demethylation of HMM resulted in a decrease in both toxic and therapeutic potencies of the metabolites. The decrease in potency was gradual initially when the number of methyl groups was reduced from 6 to 4; however, when the methyl groups were reduced to 2 or less, a precipitous decrease in potency

resulted. The primary effect of *N*-demethylation on HMM was the production of less toxic and less therapeutically potent metabolites. The decrease in therapeutic potency of the homologs as the number of methyl groups decreased and the inability of melamine to exhibit antitumor activity suggested a requisite role for the methyl groups in determining the antitumor activity of the methylmelamines.

An effect of structural change on activity was shown for the homologs that had their positional isomers included in this study. The most toxic and therapeutic positional isomers were those that possessed the most dimethyl-substituted nitrogens. A parallel finding was reported by Worzalla *et al.* (26), who noted that the metabolites of HMM found in the urine of humans and rats indicated a dimethylamino group was demethylated in preference to a monomethylamino group. This suggested that a difference in the rates of *N*-demethylation may be responsible for the difference in potencies of the isomers. Such a proposal seems logical since it is known that secondary and tertiary amines are *N*-demethylated at different rates (3).

A qualitative analysis of the results showed that, in contrast to the effect of *N*-demethylation on the toxic and therapeutic potencies, the successive removal of methyl groups from HMM did not result in substantial reductions in the efficacies of the methylmelamine homologs (Tables 2 and 3). They were more similar in magnitude than dissimilar. This finding was reflected in the similar increases in the average survival times of L1210 leukemia-bearing mice treated with equitoxic doses of the homologs (Table 4). These results suggested that the presence of a methyl group, rather than the number of groups, was the major determinant of whether the homolog functioned as an antitumor compound. Furthermore, if methyl group availability was

responsible for activity, a smooth correlation would not be expected unless each compound had similar solubility, partition coefficient, and pK. A useful structure-activity relationship would require these considerations.

One mode of action by which methyl groups could exert an antitumor action has been proposed by Bořkovec and DeMilo (2). They suggested that HMM could demethylate to form formaldehyde, a known weak alkylator. Support for this hypothesis is a study conducted with hexamethylenetetramine (methenamine) by Stevens and Lehman (16). Hexamethylenetetramine is currently used as a urinary antiseptic that has as its mechanism of action the slow release of formaldehyde in acidic solutions (21). Stevens and Lehman reported (16) that the drug exhibited an inhibitory effect on the growth of Ehrlich ascites tumors *in vivo*; the effectiveness of the drug increased as the pH of the drug solutions decreased. If the assumption is made, *a priori*, that the mechanism responsible for the antibacterial action is also responsible for the antitumor action, a mechanistic role for the methyl groups of the methylmelamine homologs would be as precursors of formaldehyde. The mechanism by which the methyl groups (formaldehyde) produce an antitumor effect may be the same as that proposed for the antitumor drug, procarbazine (NSC-77213), *i.e.*, through the transmethylation of the 7-guanine position of transfer RNA by the *N*-methyl group of the drug with inhibition of the synthesis of transfer RNA, protein, and finally DNA and RNA (12).

In contrast to hexamethylenetetramine, the formation of formaldehyde from the methylmelamines is a result of the microsomal oxygenase reactions involved in the *N*-demethylation process (4). Thus, the failure by Worzalla *et al.* (26) to show any alkylating activity by the methylmelamines when tested with 4-(*p*-nitrobenzyl)pyridine *in vitro* could have been due to the absence of microsomal enzymes from the assay protocol.

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REFERENCES

- Blum, R. H., Livingston, R. B., and Carter, S. K. Hexamethylmelamine—A New Drug with Activity in Solid Tumors. *European J. Cancer*, **9**: 195–202, 1973.
- Bořkovec, A. B., and DeMilo, A. B. Insect Chemosterilants. V. Derivatives of Melamine. *J. Med. Chem.*, **10**: 457–461, 1967.
- Brodie, B. B. The Mechanisms of Adverse Reactions. *In*: H. Raskova (ed.), *Mechanisms of Drug Toxicity*, pp. 23–47. New York: Pergamon Press, 1968.
- Ciaccio, E. I. Intimate Study of Drug Action. II: Fate of Drugs in the Body. *In*: J. R. DiPalma (ed.), *Drill's Pharmacology in Medicine*, Ed. 4, pp. 36–66. New York: McGraw-Hill, Inc., 1971.
- DeWys, W. D., Humphreys, S. R., and Goldin, A. Studies on Therapeutic Effectiveness of Drugs with Tumor Weight and Survival Time Indices of Walker 256 Carcinosarcoma. *Cancer Chemotherapy Rept.*, **52**: 229–242, 1968.
- Field, J. B., Kloetzel, M. C., and Thienes, C. H. Further Screening Studies of New Agents Against Sarcoma 180 in Mice. *Cancer Res.*, **18** (Part 2): 467–535, 1958.
- Geran, R. I., Greenberg, N. H., Macdonald, M. M., Schumacher, A. M., and Abbott, B. J. *Protocols for Screening Chemical Agents and Natural Products Against Animal Tumors and Other Biological Systems* (Third Edition). *Cancer Chemotherapy Rept.*, **3**: 1–103, 1972.
- Hirschberg, E. Patterns of Response of Animal Tumors to Anticancer Agents. *Cancer Res.*, **23** (Part 2): 521–980, 1963.
- Johnson, B. M., Grunden, E. E., and Lake, L. M. Antitumor Activity of the Homologous Methylmelamine Metabolites of the Antineoplastic Agent Hexamethylmelamine (HMM). *In*: P. Bucalossi, U. Veronesi, and N. Cascinelli (eds.), *The Eleventh International Cancer Congress*, Florence, Italy, October 20 to 26, 1974, Vol. 2, p. 140. Amsterdam: Excerpta Medica, 1975.
- Leiter, J., Abbott, B. J., Bourke, A. R., Schepartz, S. A., and Wodinsky, I. Screening Data from the Cancer Chemotherapy National Service Center Screening Laboratories. XIII. *Cancer Res.*, **23** (Part 2): 1085–1225, 1963.
- Leiter, J., Wodinsky, I., Bourke, A. R., and Schneiderman, M. A. Screening Data from the Cancer Chemotherapy National Service Center Screening Laboratories. III. *Cancer Res.*, **19** (Part 2): 488–575, 1959.
- Miller, E. Development of Procarbazine. *In*: S. K. Carter (ed.), *Proceedings of the Chemotherapy Conference on Procarbazine (Matulane: NSC-77213): Development and Application*, pp. 3–7. Washington, D. C.: United States Government Printing Office, 1971.
- Pearlman, W. M., and Banks, C. K. Substituted Chlorodiamino-5-triazines. *J. Am. Chem. Soc.*, **70**: 3726–3728, 1948.
- Reilly, H. C., Falco, E., Myron, S. A., Philips, F. S., and Stock, C. C. Sarcoma 180 Screening Data. *Cancer Res.*, **23** (Part 2): 1731–1877, 1963.
- Schepartz, S. A. Screening. *Cancer Chemotherapy Rept.*, **2**: 3–8, 1971.
- Stevens, C. D., and Lehman, R. C. Influence of pH on the Effect of Hexamethylenetetramine on the Growth of Ehrlich Ascites Tumors. *Oncology*, **28**: 411–427, 1973.
- Stock, C. C., and Buckley, S. M. Studies on the Inhibition of Sarcoma 180 in Mice. *Acta Unio Intern. Contra Cancrum*, **7**: 523–529, 1951.
- Stock, C. C., Clarke, D. A., Philips, F. S., and Barclay, R. K. Sarcoma 180 Screening Data. *Cancer Res.*, **20** (Part 2): 1–172, 1960.
- Stock, C. C., Clarke, D. A., Philips, F. S., Barclay, R. K., and Myron, S. A. Sarcoma 180 Screening Data. *Cancer Res.*, **20** (Part 2): 193–381, 1960.
- Stock, C. C., Philips, F. S., Moore, A. E., Buckley, S. M., Clarke, D. A., Barclay, R. K., and Sugiura, K. Sarcoma 180 Inhibition Screening Data. *Cancer Res.* **1** (Suppl.): 91–179, 1953.
- Tada, H. Decomposition Reaction of Hexamine by Acid. *J. Am. Chem. Soc.*, **82**: 255–263, 1960.
- Takita, H., and Didolkar, M. S. Effect of Hexamethylmelamine (NSC-13875) on Small Cell Carcinoma of the Lung (Phase II Study). *Cancer Chemotherapy Rept.*, **58**: 371–374, 1974.
- Wilson, W. L., Bisel, H. F., Cole, D., Rochlin, D., Ramirez, G., and Madden, R. Prolonged Low-Dosage Administration of Hexamethylmelamine (NSC-13875). *Cancer*, **25**: 568–570, 1970.
- Wilson, W. L., and de la Garza, J. G. Phase I Study of Hexamethylmelamine (NSC-13875). *Cancer Chemotherapy Rept.*, **48**: 49–52, 1965.
- Wilson, W. L., Schroeder, J. M., Bisel, H. F., Mrazek, R., and Hummel, R. P. Phase II Study of Hexamethylmelamine (NSC-13875). *Cancer*, **23**: 132–136, 1969.
- Worzalla, J. F., Johnson, B. M., Ramirez, G., and Bryan, G. T. *N*-Demethylation of the Antineoplastic Agent Hexamethylmelamine by Rats and Man. *Cancer Res.*, **33**: 2810–2815, 1973.
- Worzalla, J. F., Kaiman, B. D., Johnson, B. M., Ramirez, G., and Bryan, G. T. Metabolism of Hexamethylmelamine-Ring-¹⁴C in Rats and Man. *Cancer Res.*, **34**: 2669–2674, 1974.