Role of N-Methylolpentamethylmelamine in the Metabolic Activation of Hexamethylmelamine

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ABSTRACT

Hexamethylmelamine (HMM) is metabolized by rat hepatic microsomal preparations to reactive species which covalently bind to microsomal protein and to calf thymus DNA added to microsomal incubation mixtures. Covalent binding to macromolecules is dependent on the presence of molecular oxygen and reduced nicotinamide adenine dinucleotide phosphate and is catalyzed by cytochrome P-450 monoxygenases. Reduced nicotinamide adenine dinucleotide-dependent covalent binding of [methyl-14C]HMM to microsomal protein is greater than that of [ring-14C]HMM. Reduced nicotinamide adenine dinucleotide phosphate-dependent covalent binding of [ring-14C]HMM and [methyl-14C]HMM to calf thymus DNA added to microsomal incubation mixtures are approximately equal. The [ring-14C]-labeled carbinolamine intermediate in HMM demethylation, N-methylolpentamethylmelamine, covalently binds to microsomal protein and, to a much greater extent, to calf thymus DNA.

INTRODUCTION

HMM (Chart 1) is an investigational antitumor agent with demonstrated activity in combination and as a single agent against several human cancers including ovarian carcinoma, lymphomas, and small-cell carcinoma of the lung (7, 13, 21). Limited water solubility requires p.o. administration of HMM to humans. Low and variable plasma concentrations are observed following administration of HMM to patients or laboratory animals by this route (4, 11). PMM (Chart 1) was developed as a water-soluble HMM analogue for i.v. administration (10). While PMM has antitumor activity in several model test systems, it produced little evidence of cytotoxicity such as myelosuppression (or antitumor activity) in Phase I trials at doses producing limiting gastrointestinal and central nervous system toxicities (9, 14, 17, 19, 22).

There is evidence that metabolism of HMM is required for antitumor activity (29), but the mechanism for antitumor activity is not known. HMM and PMM are extensively metabolized to demethylated metabolites in vivo and in vitro (4, 16, 32, 33). Neither HMM nor its demethylated metabolites have alkylation activity as measured by reaction with p-nitrobenzylpyridine (33) or by covalent binding of radiolabeled HMM and PMM to macromolecules in the absence of an enzymatic activating system (5, 6). We recently demonstrated that HMM and PMM are activated by hepatic microsomes to reactive intermediates which covalently bind to tissue macromolecules (5, 6). Our studies led us to suggest that MPMM (Chart 1), the carbinolamine intermediate formed during hepatic N-demethylation of HMM, might be involved in the covalent binding of HMM to microsomal macromolecules (6). MPMM has been identified as an in vitro HMM metabolite (16). We now report further studies on the metabolic activation of HMM to reactive species which covalently bind to macromolecules and on covalent binding of [ring-14C]MPMM to microsomal protein and calf thymus DNA.

MATERIALS AND METHODS

Chemicals. HMM, PMM hydrochloride, and N,N,N,N,N'-TMM hydrochloride were supplied by Leonard H. Kedda, Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, Md. [ring-14C]HMM, specifically [ring-2,4,6-14C]HMM (13.0 mCi/mmol), and [methyl-14C]HMM, specifically [2,4,6-N(CH3)3-14C]HMM (8.4 mCi/mmol) were supplied by Dr. Robert Engel, Chemical Resources Section, Pharmaceutical Resources Branch, Division of Cancer Treatment, National Cancer Institute. Chemical and radiopurity of the labeled melamines were greater than 99% as determined by thin-layer chromatography (indicator silica gel plates, ethyl acetate:petroleum ether, 90:10, mobile solvent) and GC (10% Carbowax 20 M:2% potassium hydroxide column with nitrogen:phosphorus detector) analysis (2, 4). Fluorescence quenching and scintillation counting of eluted thin-layer chromatography fractions only detected materials with Rf values of authentic HMM, 0.70. GC retention times of the melamines were identical to the value obtained with authentic HMM, using chromatographic conditions described elsewhere (2, 4). The synthesis of [ring-2,4,6-14C]MPMM will be reported in detail elsewhere. Briefly, [ring-2,4,6-14C]cyanuric chloride (7.14 mCi/mmol; Pathfinder Chemicals, St. Louis, Mo.) was diluted with 2 parts unlabeled cyanuric chloride (Aldrich Chemical Co., Milwaukee, Wis.) and aminated with dimethylamine to provide [ring-2,4,6-14C]-2-chloro-4,6-bis(dimethylamino)-1,3,5-triazine in 97% yield. Reaction of the labeled monochlorotriazine with methylamine provided [ring-2,4,6-14C]-2-methylaminobenzene (1,3,5-triazine) and dimethylaminobenzene (97% yield). Reaction of [ring-2,4,6-14C]MPMM with aqueous formaldehyde provided the [ring-2,4,6-14C]MPMM in 57% yield (2.4 mCi/mmol). Melting points, nuclear magnetic resonance spectra, and mass spectrometric spectra of labeled intermediates and the final product were identical to literature values reported for unlabeled intermediates and unlabeled final product prepared in optimizing the reaction sequence. Glucose-6-phosphate dehydrogenase and NADP* were purchased from Boehringer-Mannheim Biochemicals, Indianapolis, Ind. Phenobarbital, glutathione, calf thymus DNA, and sodium dodecyl sulfate were purchased from Sigma Chemical Co., St. Louis, Mo. SKF-525A was purchased from Smith Kline and French, Philadelphia, Pa. All other solvents and reagents were reagent or chromatographic grade.

Animals, Tissue Preparation, and Incubation Conditions for HMM Binding Studies. Male Sprague-Dawley rats (150 to 250 g; Mayo Clinic) were maintained on laboratory chow and water ad libitum.

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3 The abbreviations used are: HMM, hexamethylmelamine; PMM, penta- melamine; MPMM, N-methylolpentamethylmelamine; N,N,N,N,N'-TMM, N,N,N,N,N'-tetramethylmelamine; GC, gas chromatography; SKF-525A, 2-de- methylaminobenzyl-2,2-diphenyl valerate hydrochloride; HPLC, high-performance liquid chromatography; MS, mass spectrometry; DAB, N,N-dimethyl-4-aminobenzene.

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Metabolic Activation of HMM

Microsomes were prepared by the method of Ernster et al. (15). Protein was determined by the method of Lowry et al. (24). Microsomal incubations (3 ml total volume) contained the following components present in the indicated final concentrations: NADP⁺ (0.5 mM); glucose 6-phosphate (25 mM); glucose-6-phosphate dehydrogenase (0.5 units/ml); MgCl₂ (5 mM); microsomal protein (2.0 mg/ml); and substrate (0.25 mM added in 50 μl of acetone). For DNA binding studies, calf thymus DNA was first dissolved in 0.9% NaCl solution (10 mg/ml), and 0.3 to 0.5 ml was added to microsomal incubation mixtures.

Incubations (3 ml total volume) contained the following components:
- 6-phosphate (25 mM); glucose-6-phosphate dehydrogenase (0.5 units/ml); MgCl₂ (5 mM); microsomal protein (2.0 mg/ml); and substrate (0.25 mM added in 50 μl of acetone). For DNA binding studies, calf thymus DNA was first dissolved in 0.9% NaCl solution (10 mg/ml), and 0.3 to 0.5 ml was added to microsomal incubation mixtures.

Radiolabeled HMM was present at a final activity of 1500 dpm/nmol. Cocktail and drug were added to the incubation flask (25 ml), and the reaction was started by addition of microsomes. Reactions were carried out at 37°C for 0 to 60 min. Reaction mixtures were cooled in an ice bath, and microsomal protein was isolated by addition of ice-cold acetone (3 ml) followed by low-speed centrifugation of denatured protein.

Methods for removal of noncovalently bound radiolabel from protein and determination of covalent binding have been published (1, 6). Briefly, the protein pellet was washed repeatedly with aqueous methanol (80%) and ethanol-ether (1:4, v/v) until further radioactivity could not be extracted. This procedure usually consisted of 4 washes with 80% methanol followed by 3 washes with ethanol-ether or until no radioactivity was detected in pellet supernatants. The extracted pellet dissolved in 0.5 ml 1 N sodium hydroxide and heated to 60°C in a water bath for 15 min, and two 20-μl aliquots were removed for protein determination. The remaining 460 μl were transferred to a scintillation vial to which 1.5 ml water and 15 ml scintillation fluid were added. Radioactivity was collected for background counting and expressed as described in the text. For isolation of DNA from reaction mixtures, incubations were terminated by addition of 6 ml phenol saturated with 0.1 M sodium acetate (pH 6). The mixture was shaken for 15 min and centrifuged, and the phenolic layer was discarded. The extraction procedure was repeated. To the aqueous phase was added 2 times the volume of ice-cold 95% ethanol, and samples were allowed to sit overnight at -18°C. The following morning, samples were centrifuged, and the DNA pellet was washed sequentially with ethanol, ethanol-ether, and ethyl acetate until no radioactivity was detected in the washes to remove nonreversibly bound radioactivity. DNA was hydrolyzed by heating at 60°C for 1 hr in 0.3 ml 0.3 N perchloric acid. An aliquot was removed for analysis of DNA by the diphenylamine method (8), the remaining volume was added to scintillation vials, and radioactivity was determined as expressed in the text.

Chart 1. Structures of HMM, PMM, and MPMM.

![Chart 1](chart1.jpg)

Animals, Tissue Preparation, and Incubation Conditions for Binding Studies with [ring-14C]MPMM. Microsomal incubations were as described above for HMM binding studies, except NADP⁺ was not added to incubation mixtures. When binding to DNA was studied, the reaction mixture consisted of 1 to 5 mg calf thymus DNA in 0.1 M Tris buffer (pH 7.4) without other components of the incubation mixture. The final concentration of MPMM was 0.25 mM (1500 dpm/nmol). Radioactivity bound to microsomal protein and DNA was determined as described for HMM binding studies.

HPLC Analysis of MPMM and PMM. Aliquots of incubation mixtures (buffer alone, buffer and cofactors, or buffer, cofactors, and microsomes) containing MPMM were extracted with ethyl acetate or added to ice-cold acetone and precipitated protein removed by centrifugation. Aliquots of ethyl acetate extract or deproteinized aqueous:acetone were injected for HPLC analysis. A reverse-phase C18 column (10 μm; Waters Associates, Milford, Mass.) and acetonitrile:0.01 M ammonium formate (50:50) mobile solvent provided chromatographic separation of MPMM and PMM (16), and detection was by UV absorbance at 254 nm.

Hydrolysis of Protein and DNA Pellets and Identification of Hydrolysis Products. HMM and MPMM were incubated with microsomal preparations and microsomal preparations containing DNA under conditions described for binding studies. Protein and DNA were isolated and washed as described above. The pellets were hydrolyzed in 1 N hydrochloric acid (60°C) for 30 min, and the pH was adjusted to 10 with 5 N sodium hydroxide. The hydrolysates were extracted with toluene, the organic extract evaporated by a gentle stream of nitrogen, and the residue taken up in toluene for GC or GC-MS analysis.

Nitrogen:phosphorus GC analyses of HMM, PMM, and their demethylated metabolites with 10% Carbowax:2% potassium hydroxide columns have been published (2, 4). Retention times of materials eluted from hydrolysis extracts were compared with retention times of authentic HMM, PMM, and N²,N²,N⁴,N⁴-TMM. GC-MS analyses of hydrolysis extracts were carried out on an LKB 2091 GC/MS equipped with the same analytical column packing used in the GC assay. Electron impact mass spectra (70 eV) were compared to spectra obtained with authentic PMM and N²,N²,N⁴,N⁴,N⁵-TMM.

RESULTS

Covalent Binding of HMM Metabolite(s) to Microsomal Protein in Calf Thymus DNA. Incubation of [ring-14C]- or [methyl-14C]-HMM with rat liver microsomal preparations resulted in NADPH-dependent covalent binding of radiolabel to microsomal protein during 60-min incubations (Chart 2). Greater covalent binding to microsomal protein was observed when [methyl-14C]-HMM was added to incubation mixtures than when [ring-14C]-HMM was added to incubation mixtures (Chart 2). In addition to being dependent on the presence of NADPH, covalent binding required molecular oxygen and fresh microsomes (Table 1A). Pretreatment of rats with phenobarbital significantly increased total NADPH-dependent covalent binding of labeled HMM of microsomal protein (Table 1A). Addition of 0.1 mM SKF-525A to incubation mixtures or incubation in

![Chart 2](chart2.jpg)
Table 1
Characterization of covalent binding of HMM to microsomal protein and calf thymus DNA

<table>
<thead>
<tr>
<th>Incubation conditions</th>
<th>% of Control (30-min incubation)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A. Microsomal protein (2 mg/ml)</strong></td>
<td></td>
</tr>
<tr>
<td>Complete</td>
<td>100</td>
</tr>
<tr>
<td>~NADP</td>
<td>4 ± 1&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>~O&lt;sub&gt;2&lt;/sub&gt; (100% N&lt;sub&gt;2&lt;/sub&gt;)</td>
<td>14 ± 1&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Boiled microsomes</td>
<td>5 ± 1&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>CO&lt;sub&gt;2&lt;/sub&gt;:O&lt;sub&gt;2&lt;/sub&gt; (80:20)</td>
<td>61 ± 6&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>SKF-525A</td>
<td>46 ± 2&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Phenobarbital (80 mg/kg i.p.; 3 days)</td>
<td>164 ± 15&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>B. Calf thymus DNA (3 mg/ml)</strong></td>
<td></td>
</tr>
<tr>
<td>Complete</td>
<td>100</td>
</tr>
<tr>
<td>~NADP</td>
<td>3 ± 1&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>~O&lt;sub&gt;2&lt;/sub&gt; (100% N&lt;sub&gt;2&lt;/sub&gt;)</td>
<td>15 ± 4&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Boiled microsomes</td>
<td>6 ± 1&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>CO&lt;sub&gt;2&lt;/sub&gt;:O&lt;sub&gt;2&lt;/sub&gt; (80:20)</td>
<td>41 ± 1&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>SKF-525A</td>
<td>64 ± 5&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Phenobarbital (80 mg/kg i.p., 3 days)</td>
<td>184 ± 13&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Substrate concentration, 0.25 mM.<br><sup>b</sup> Mean ± S.E. for 3 or more experiments.<br><sup>c</sup> p < 0.05.

an 80:20 (v/v) carbon monoxide:air atmosphere significantly reduced NADPH-dependent covalent binding to microsomal protein (Table 1A).

Addition of exogenous calf thymus DNA to microsomal incubation mixtures containing [ring-<sup>14</sup>C]- or [methyl-<sup>14</sup>C]-HMM led to covalent binding of radiolabel to isolated DNA during 60-min incubations. NADPH-dependent covalent binding of [ring<sup>-14</sup>C]- and [methyl-<sup>14</sup>C]-HMM to DNA were not statistically different (Chart 3). Covalent binding to DNA was dependent on the presence of NADPH, molecular oxygen, and fresh microsomes (Table 1B). Phenobarbital pretreatment of rats increased NADPH-dependent covalent binding of labeled HMM to calf thymus DNA (Table 1B), while addition of 0.1 mM SKF-525A or incubation in an 80:20 (v/v) carbon monoxide:air atmosphere reduced NADPH-dependent covalent binding of labeled HMM to calf thymus DNA.

Binding of [ring-<sup>14</sup>C]-MPMM to Microsomal Protein and DNA. Incubation of [ring-<sup>14</sup>C]-MPMM with microsomal protein (in the absence of an NADPH-generating system) or with calf thymus DNA resulted in covalent binding of radiolabel to these macromolecules. Covalent binding to protein and DNA increased during 60-min incubation periods (Chart 4), and covalent binding to DNA was much greater than to microsomal protein (Chart 4) on a mg basis. When MPMM was preincubated (0 to 30 min) in the incubation buffer prior to addition of protein or DNA, covalent binding was reduced. The half-life of MPMM in buffer containing cofactors (NADPH, MgCl<sub>2</sub>, glucose 6-phosphate, glucose-6-phosphate dehydrogenase) and in buffer with microsomal protein was 40 and 33 min, respectively, as determined by HPLC analysis. Disappearance of MPMM was associated with appearance of PMM in HPLC chromatograms.

Identification of Hydrolysis Products of Microsomal Protein and DNA Drug Adducts. Acid and base hydrolysis of washed microsomal protein pellets obtained from incubations containing HMM followed by extraction (toluene) and nitrogen:phosphorous GC analysis indicated the presence of PMM, and a lesser amount of N<sub>2</sub>,N<sub>2</sub>,N<sub>6</sub>,N<sub>6</sub>-TMM (Chart 5 A). PMM and N<sub>2</sub>,N<sub>2</sub>,N<sub>4</sub>,N<sub>6</sub>-TMM were detected only in extracts from microsomal incubations containing MPMM.
incubation mixtures containing an NADPH-generating system (Chart 5A). Identical results were obtained following acid or base hydrolysis of washed DNA pellets (data not shown). Only PMM was observed following hydrolysis of protein or DNA pellets obtained from incubations containing MPMM (Chart 5B). Identity of PMM and \( \text{N2,N2,N4,N6-TMM} \) in all extracts was verified by GC-MS analysis. Mass spectra of PMM and \( \text{N2,N2,N4,N6-TMM} \) obtained by GC-MS analyses were characterized by the presence of strong molecular ions at \( m/z \) 196 and 182, respectively, and were identical to mass spectra of authentic PMM and \( \text{N2,N2,N4,N6-TMM} \).

**DISCUSSION**

We have shown that HMM is metabolically activated by the hepatic microsomal preparations to reactive intermediates which covalently bind to microsomal protein and calf thymus DNA. Enzymatic activation requires molecular oxygen and NADPH, and studies with phenobarbital, SKF-525A, and carbon monoxide suggest involvement of cytochrome P-450 monoxygenases. To assist in determination of the species responsible for covalent binding, we carried out binding studies with \([\text{ring-14C}]\) and \([\text{methyl-14C}]\) HMM and compared the NADPH-dependent covalent binding of each substrate to microsomal protein and calf thymus DNA. NADPH-dependent covalent binding to microsomal protein was greater for \([\text{methyl-14C}]\) HMM than for \([\text{ring-14C}]\) HMM. This suggests that 2 species may be involved in covalent binding to microsomal protein. Binding of \([\text{ring-14C}]\) HMM demonstrates that one reactive intermediate contains the triazine ring system. The greater binding of \([\text{methyl-14C}]\) HMM indicates that a reactive methyl moiety such as formaldehyde binds to microsomal protein, and to a greater extent than the ring-containing intermediate. There was no significant difference in the NADPH-dependent covalent binding of \([\text{ring-14C}]\) HMM and \([\text{methyl-14C}]\) HMM to calf thymus DNA added to microsomal incubation mixtures. Equivalent binding of \([\text{ring-14C}]\) and \([\text{methyl-14C}]\) HMM to DNA is best explained by binding of a species containing the entire HMM molecule. Preferential binding of a released methyl moiety would lead to greater binding of the methyl-labeled substrate (as with microsomal protein) while preferential binding of demethylated ring metabolites would lead to greater binding of the ring-labeled substrate.

The only known pathway of HMM metabolism involving hepatic microsomal monoxygenases is N-demethylation, catalyzed by cytochrome P-450 (3, 16, 28). The products of the oxidation are formaldehyde and PMM, formed by spontaneous decomposition of the initial carbinoamine C-oxidation product, MPMM (3, 16, 28). We synthesized \([\text{ring-14C}]\) MPMM and found that it does bind covalently to calf thymus DNA in the absence of a fortified incubation system. It also binds, but to a much lesser extent, to microsomal protein in the absence of an NADPH-generating system. While the number of binding sites on a molecule of RNA and microsomal protein may differ, these data are consistent with our hypothesis that a reactive methyl moiety is responsible for most of the NADPH-dependent covalent binding of HMM to protein while MPMM is the species responsible for binding to DNA.

N-Demethylation has not historically been viewed as a drug metabolism pathway leading to the generation of reactive intermediates (25, 30). In most cases, the initial oxidation products (carbinoamines) decompose spontaneously to the corresponding aldehydes and dealkylated amines (25, 30). However, substituents adjacent to the nitrogen atom which delocalize the lone pair nitrogen electrons can stabilize carbinoamines. In addition to MPMM (16), stable carbinoamine metabolites have been isolated as metabolites to a number of drugs including benzamides (18), carbamates (12), and the antitumor agent procarbazine (31). Although not formed metabolically, several stable pyrrolo(1,4)benzodiazepine antitumor antibiotics contain a carbinoamine moiety in the benzodiazepine ring nucleus (3, 23). Recent studies have shown that carbinoamine intermediates formed during N-demethylation reactions can lead to reactive species capable of binding to nucleo- moieties. Nguyen et al. (26, 27) and Ziegler et al. (34) demonstrated that microsomal demethylation of nicotine and methapyriline leads to reactive intermediates which can be trapped as cyanide adducts (or decomposition products) consistent with formation of initial carbinoamine metabolites. In studies with DAB, Ketterer et al. (20) isolated a glutathione adduct apparently derived from a reactive carbinoamine intermediate formed during demethylation of the monodemethylated metabolite of DAB. The antitumor antibiotics tomaymycin, sibiromycin, and anthramycin bind covalently to DNA, and binding appears to occur via the carbinoamine moiety (3, 23). Our studies with HMM and MPMM directly implicate a carbinoamine intermediate in the metabolic activation of a xenobiotic agent as measured by covalent binding to macromolecules.

We suggest that the NADPH-dependent covalent binding of HMM (via MPMM) may be explained by formation of a reactive iminium species from MPMM (as illustrated in the scheme shown in Chart 6). MPMM preferentially decomposes to the demethylated metabolite (PMM) and formaldehyde. The carbinoamine may also decompose to an iminium ion which in turn reacts with macromolecular nucleophiles (Chart 6, x). Formation of iminium species from carbinoamine intermediates has been suggested by Nguyen et al. (26, 27) and Ziegler et al. (34) in their studies on the activation of nicotine and methapyriline and by Ketterer et al. (20) in their studies with DAB. The activation pathway shown in Chart 6 is further suggested by results of our hydrolysis studies. Hydrolysis of an adduct formed from the iminium species and a nucleophilic site on a...
presence of a hepatic activating system, submitted for publication. of colony formation by human tumor cells plated on plastic is dependent on the amounts of PMM. GC chromatograms from extracts of extracts. Minor quantities of N2,N2,N4,N%-TMM were observed in the GC chromatograms relative to N2,N2,N4-tetramethylmelamine (4). The extent of covalent binding of PMM is significantly less than that of covalent binding of HMM is further demethylated by microsomes opposed to N2,N2,N4-tetramethylmelamine (4). The extent of covalent binding of PMM is significantly less than that observed for HMM (5, 6) consistent with the small amounts of N2,N2,N4,N%-TMM observed in the GC chromatograms relative to the amounts of PMM. GC chromatograms from extracts of incubations containing only MPMM contained only PMM.

The role of metabolic activation in the antitumor activity of HMM is not known. It has been suggested previously that HMM requires metabolism in order to express its antitumor activity (5, 6, 16, 28). We have found that HMM is relatively inactive against several human tumor cell lines in the human tumor stem cell colony-forming assay, except when coincubated with a hepatic activating system. There are other antitumor agents, most notably cyclophosphamide, which require oxidative metabolic activation in order to express their activity. Finally, the poor clinical results with PMM correlate with our earlier findings that PMM is a much poorer alkylating agent than HMM as measured by NADPH-dependent covalent binding to tissue macromolecules (5, 6).

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