Metabolism of Hexamethylene Bisacetamide and Its Metabolites in Leukemic Cells

Merrill J. Egorin, Stuart W. Snyder, Adam S. Cohen, Eleanor G. Zuhowski, Babu Subramanyam, and Patrick S. Callery

ABSTRACT

We investigated whether leukemic cell lines could convert hexamethylene bisacetamide (HMBA) to any of the metabolites previously identified and quantified in the urine and plasma of patients treated with HMBA. After 5–7 days of incubation with 1–2 mM HMBA, HL60 human promyelocytic leukemic cells, L1210 and P388 murine lymphoblastic leukemic cells, and Friend murine erythroleukemia cells contained 4 of the previously identified metabolites of HMBA. Gas chromatography/mass spectrometry confirmed the presence of N-acetyl-1,6-diaminohexane (NADAH), 1,6-diaminohexane (DAH), 6-aminohexanoic acid (AcHA), and 6-aminohexanoic acid (AmHA). Gas chromatography with nitrogen-phosphorus selective detection was used to quantify cellular concentrations of each metabolite. Cellular concentrations of AmHA and DAH were greater than those of NADAH and AcHA but no concentration of a metabolite exceeded that of HMBA. Metabolites were not detected in media from cells incubated with HMBA. Friend murine erythroleukemia cells that were resistant to HMBA contained only HMBA and NADAH. Moreover, the concentrations of NADAH in Friend murine erythroleukemia cells that were resistant to HMBA were less than those in the other cell lines studied. HL60 cells accumulated HMBA rapidly. NADAH, DAH, AcHA, and AmHA appeared sequentially in HL60 cells that were incubated with HMBA. NADAH appeared very rapidly, but concentrations of DAH were ≥ those of NADAH by 8 h. AcHA and AmHA were not detected in cells before 24–48 h of incubation with HMBA. HL60 cells incubated with individual HMBA metabolites were able to accumulate each compound and to interconvert some: cells incubated with NADAH also contained DAH, AcHA, and AmHA; cells incubated with AcHA also contained low concentrations of AmHA; cells incubated with DAH also contained AmHA; and cells incubated with AmHA contained no other HMBA metabolites. HMBA was not present in cells incubated with any of its known metabolites. These results document the ability of various leukemic cells to metabolize HMBA, indicate the unidirectional catabolism of that compound, and may have implications as to its mechanism of action.

INTRODUCTION

HMBA (NSC 95580) induces in vitro morphological and functional differentiation of murine and human leukemic and solid tumor cell lines (1–13). Among the class of agents that induce differentiation of tumor cells (14–19), HMBA has a number of characteristics which render it one of the most interesting and of greatest potential clinical use (20). Although HMBA has been introduced into clinical trials (20–24), the mechanism of action of HMBA-induced differentiation remains undefined. Moreover, the in vitro activity of HMBA has not been duplicated in a variety of in vivo systems (20). We have recently utilized GC/MS to identify 5 metabolites of HMBA in the urine of patients treated with HMBA (25). These metabolites included the major metabolite, AcHA; the monodeacetylated product, NADAH; the bis-deacetylated diamine, DAH; and the amino acid, AmHA with its lactam, caprolactam (Fig. 1). Subsequently, we have developed suitable GC methodology (26) and have quantified the concentrations of each of these metabolites in the plasma and urine of patients receiving various doses of HMBA (26, 27). Our documentation of the presence of these metabolites and the quantification of pharmacologically relevant concentrations of each compound allowed us to investigate the potential of each metabolite to induce cellular differentiation and to assess what effect these compounds had on HMBA-induced differentiation (28). In concurrence with the recently published studies of Meilhoc et al. (29), we found NADAH capable of inducing differentiation of HL60 human promyelocytic leukemic cells, but unlike those studies (29), we found NADAH to be a more potent inducer of differentiation than was HMBA (28). Moreover, when combined in pharmacologically relevant concentrations, we observed that AcHA, a compound incapable of inducing differentiation by itself, potentiated the induction of differentiation by HMBA and NADAH (28). Additional studies in our laboratory have begun to characterize the enzymes involved in the catabolism of HMBA and its metabolites (30). We have shown that conversion of NADAH to AcHA is catalyzed by monoamine oxidase, and not by microsomal enzymes, and that the conversion of DAH to AmHA is catalyzed by diamine oxidase (30). Although Meilhoc et al. (29) have shown Friend murine erythroleukemia cells to deacetylate HMBA to NADAH and DAH sequentially, and earlier studies of HMBA (4) provided evidence for cellular deacetylation of this compound, the complete characterization of HMBA catabolism by cells remains undefined. In the belief that definition of the cellular metabolism of HMBA would provide important clues about the mechanism of action of the drug and would complement our clinical pharmacological results, we undertook the studies described in this manuscript.

MATERIALS AND METHODS

Reagents. HMBA was supplied by the Investigational Drug Branch, National Cancer Institute, Bethesda, MD, as a 30-mg/ml solution in aqueous 0.154 M NaCl. DAH, AcHA, AmHA, 1,2-diphenylethylamine, cadaverine, 2,2,2-trifluoroethanol, trifluoroacetic anhydride, and acetic anhydride were purchased from Sigma Chemical Co. (St. Louis, MO). NADAH was synthesized from DAH and acetic anhydride as described previously (25).

Cell Lines. HL60 human leukemia cells, a line derived from peripheral blood leukocytes of a patient with acute progranulocytic leukemia (31), were maintained in vitro by serial culture twice weekly in RPMI Medium 1640 (GIBCO, Grand Island, NY) containing penicillin (50 units/ml), streptomycin (50 μg/ml), 1-glutamine (2 μmol/ml), and 15% (v/v) heat-inactivated fetal bovine serum (GIBCO) (Medium A). Cells
were maintained at 37°C in an atmosphere of 5% CO₂, 95% air, and 95% humidity. Under these conditions, the cell population had a doubling time of approximately 24 h and achieved a maximum cell density of 2-3 × 10⁶/ml.

L1210 murine leukemia cells (32, 33) were maintained in vitro by serial culture twice weekly in Medium A at 37°C in an atmosphere of 5% CO₂, 95% air, and 95% humidity. Under these conditions, the cell population had a doubling time of approximately 14-18 h and achieved a maximum cell density of 1.5-2 × 10⁶/ml.

P388 murine leukemia cells (34, 35) were maintained in vitro by serial culture twice weekly in Medium A at 37°C in an atmosphere of 5% CO₂, 95% air, and 95% humidity. When maintained at 37°C in an atmosphere of 5% CO₂, 95% air, and 95% humidity, the cell population had a doubling time of approximately 12-14 h and achieved a maximum cell density of 2-2.6 × 10⁶ cells/ml.

MEL (36) or MELR (37) were maintained in vitro by serial twice-weekly culture in Medium A at 37°C in an atmosphere of 5% CO₂, 95% air, and 95% humidity. Under these conditions, sensitive and resistant cell populations had doubling times of approximately 12 and 17 h, respectively, and both achieved maximum cell densities of approximately 3 × 10⁶/ml.

Incubation of Cells with HMBA and Metabolites. On each of the 2 days preceding institution of an experiment, cell cultures were diluted 1:4 with fresh medium. Experiments were begun by resuspending cells to a final concentration of approximately 1-2 × 10⁶/ml in Medium A that contained the desired concentration of HMBA or metabolite. These cells were incubated in the presence of HMBA or a metabolite for specified times at 37°C in an atmosphere of 5% CO₂, 95% air, and 95% humidity. Experiments with incubation times of 30 min-24 h used initial cell suspensions of 10⁶/ml after control experiments demonstrated no cell density-related differences in metabolism of HMBA over the specified times. Similarly, experiments with incubation times of 5-30 min used initial cell suspensions of 10⁶/ml after appropriate controls ruled out cell density-related changes in drug metabolism over the specified times.

At completion of incubation, cell suspensions were transferred to conical centrifuge tubes, and cells were sedimented by centrifugation at 1150 × g for 10 min. Medium supernatants and respective cell pellets were stored frozen at −20°C until analysis.

Analysis of HMBA and Metabolites. Medium supernatants were thawed and analyzed directly. Before being analyzed, cell pellets were freeze-thawed 3 times and then sonicated for 10 sec with a Bronwill (Rochester, NY) Biosonik III sonicator set at 30. HMBA concentrations were assessed by GC with our modification (21) of the method of Kelley et al. (38). Concentrations of NADAH, DAH, AcHA, and AmHA were assessed by GC with our previously described method (26). The quantitative aspects of each of these assays with regard to reproducibility and sensitivity have been published previously (21, 26).

Gas Chromatography/Mass Spectrometry Confirmation of Metabolite Structures. Formation of trifluoroacetyl derivatives of amino functional groups and 2,2,2-trifluoroethyl ester derivatives of carboxylic acid groups was accomplished by dissolution of a portion of the freeze-thawed and sonicated cell pellet in trifluoroacetic anhydride (0.2 ml) followed by addition of 2,2,2-trifluoroethanol (0.1 ml) and then heating for 30 min at 50-60°C (25, 26). After this derivatization, reaction mixtures were evaporated to dryness under a stream of nitrogen, and the residue was dissolved in ethyl acetate for GC/MS analysis. A Hewlett-Packard 5970 gas selective detector (Hewlett-Packard, Palo Alto, CA) was used to obtain electron ionization spectra. Chromatography was carried out on a Hewlett-Packard 5890 gas chromatograph equipped with a 15-m (0.25 mm inside diameter) Durabond 1701 capillary column (J & W Scientific, Inc., Rancho Cordova, CA). Helium was used as carrier gas and maintained a column head pressure of 40 kPa. After a 1.5-min solvent delay, the column was heated at 15°C/min from a starting temperature of 80°C to a final temperature of 260°C. Spectra were obtained with an ionization voltage of 70 eV at a scan rate of 429 atomic mass units/sec over a mass range of 50-500.

RESULTS

After 5 to 7 days of incubation, each cell line studied contained HMBA concentrations 1.1 to 1.9 times those present in the surrounding medium (Table 1). With the exception of MELR cells, each of the cell lines studied contained all of the metabolites that had previously been identified in the urine of patients treated with HMBA (Table 1) (25). More specifically, after 5 to 7 days of incubation with 1 to 2 mM HMBA, HL60, L1210, P388, and MEL cells all contained NADAH, DAH, AcHA, and AmHA. Structural confirmation of each cellular metabolite was obtained by GC/MS and direct comparison to authentic standards and relative retention time values (25). Parallel control experiments ruled out the possibility of artificial results arising from endogenous materials or sample preparation and derivatization procedures.

Table 1: Concentrations of HMBA and metabolites in leukemic cells incubated with HMBA

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Supernatant</th>
<th>Cellular concentration (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HL60</td>
<td>1.52*</td>
<td>2.31 0.22 1.00 0.21 1.62</td>
</tr>
<tr>
<td>L1210</td>
<td>1.32</td>
<td>2.27 0.23 0.83 0.30 0.49</td>
</tr>
<tr>
<td>P388</td>
<td>1.26</td>
<td>2.13 0.15 0.94 0.26 0.29</td>
</tr>
<tr>
<td>MEL</td>
<td>1.01</td>
<td>1.95 0.24 0.20 0.32 1.92</td>
</tr>
<tr>
<td>MELR</td>
<td>1.60</td>
<td>1.04 0.13 ND ND ND</td>
</tr>
<tr>
<td>MELR</td>
<td>1.27</td>
<td>1.42 0.11 ND ND ND</td>
</tr>
</tbody>
</table>

* Mean of 2-4 determinations. In all cases, SD was ≤ 32% of the mean.

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CELLULAR METABOLISM OF HMBA AND METABOLITES

GC analysis of control medium revealed no materials with chromatographic characteristics similar to those of HMBA or any of its metabolites. The metabolite profile of MELR cells was very different from that of the cell lines just described. MELR cells contained only one metabolite of HMBA, that being NADAH. Moreover, the concentration of NADAH in MELR cells was less than that in MEL cells. MELR cells did not appear capable of metabolizing NADAH by deacetylation to DAH or by oxidation to AcHA.

When the temporal aspects of cellular accumulation of HMBA and metabolites were examined, HL60 cells were observed to accumulate HMBA rapidly (Table 2). Cellular deacetylation of HMBA to NADAH also occurred quickly, although intracellular steady-state concentrations of NADAH were not achieved until approximately 24 h (Table 2). The cellular concentrations of NADAH observed at 24, 48, 120, 144, and 168 h in these time course experiments were not only consistent from day to day, but also were similar to those measured in earlier experiments which used a 7-day incubation of HL60 cells with HMBA to characterize which cellular HMBA metabolites would be present (Table 1). DAH appeared in HL60 cells more slowly than did HMBA and NADAH with the earliest measured concentrations of DAH occurring at 1 h (Table 2). AcHA and AmHA required much longer to be detected in cells, but, by 24 h, AmHA and, by 48 h, AcHA had achieved concentrations greater than or equal to those of NADAH (Table 2). At no time did cellular concentrations of any metabolite exceed those of HMBA and at no time were NADAH, DAH, AcHA, or AmHA detected in the incubation medium (26).

When incubated in medium containing individual HMBA metabolites, HL60 cells were capable of taking up each HMBA metabolite (Table 3). Moreover, once taken up, HMBA metabolites were further interconverted by HL60 cells to other known metabolites of HMBA (Table 3). Cells incubated with NADAH also contained DAH, AcHA, and AmHA, implying successive deacetylation and oxidation (Table 3). Cells incubated with DAH achieved cellular concentrations of DAH approximately one and a half times those present in the surrounding incubation medium (Table 3). In addition to DAH, these cells contained large concentrations of AmHA, the product of diamine oxidase-catalyzed metabolism of DAH (30). Cells incubated with AcHA contained concentrations of this metabolite approximately equal to those present in the surrounding incubation medium (Table 3). In addition to AcHA, cells incubated with AcHA also contained relatively low concentrations of AmHA (Table 3). These concentrations of AmHA in cells incubated with AcHA were far less than the concentrations of AmHA present in cells incubated with HMBA, NADAH, or DAH (Tables 1 and 3). Finally, cells incubated with AmHA contained concentrations of that metabolite approximately 3 times those present in the incubation medium (Table 3) and contained none of the other known metabolites of HMBA. HMBA was not present in the cell pellets or in the medium associated with cells incubated with any HMBA metabolite (Table 3).

DISCUSSION

Despite extensive documentation and characterization of the ability of HMBA to induce cellular differentiation of murine and human solid and leukemia cell lines (1-13), and the initiation of clinical trials with this agent (21-24), a number of aspects of the pharmacology of the compound, including its mechanism of action, remain unknown. Recent studies from our laboratory have documented and characterized the in vivo biotransformation of HMBA with resultant production of at least 4 metabolites (25-27). Having identified NADAH, DAH, AcHA, and AmHA as metabolites of HMBA, we have continued to pursue the issues of how these materials are produced and interconverted (30). We have also considered potential roles for these materials in the mechanism of action of HMBA and in the disparity between the in vitro activity of the drug and lack thereof in vivo (28). The results presented in the current manuscript clearly document the ability of a number of leukemic cell lines to take up HMBA and to convert it to the same metabolites identified in the plasma and urine of patients treated with HMBA. A number of aspects of this metabolite production are of note. The presence of easily detectable concentrations of each metabolite in cells that had been incubated with HMBA and the lack of detectable concentrations of those metabolites in incubation medium from which the cells were isolated implies a cellular mechanism for accumulating each metabolite or some means by which the various metabolites are trapped intracellularly once they are formed. The ability of each metabolite to be taken up by cells that have been incubated with individual metabolites provides direct evidence for the former mechanism. The fact that DAH and AmHA represent major intracellular metabolites yet are present in trivial concentrations in the urine and plasma of patients treated with HMBA (26,

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Concentrations of HMBA and metabolites in HL60 cells after varying periods of incubation with HMBA</th>
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<tbody>
<tr>
<td><strong>HMBA</strong></td>
<td><strong>Time (h)</strong></td>
</tr>
<tr>
<td>0.083</td>
<td>1.99&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.17</td>
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<tr>
<td>1</td>
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<tr>
<td>144</td>
<td>1.23</td>
</tr>
<tr>
<td>168</td>
<td>1.60</td>
</tr>
</tbody>
</table>

<sup>a</sup> Concomitant concentration of HMBA in medium from which cells were harvested. The initial HMBA concentration was 2 mM in cultures of 150 ml. At no time were NADAH, DAH, AcHA, or AmHA detected in medium (26).

<sup>b</sup> Mean of 2-8 determinations. In all cases, SD was ≤ 33% of the mean.

<sup>c</sup> ND, not detectable.
27) is also consistent with the intracellular sequestration of these metabolites once they are formed. Although we have previously shown monoamine oxidase to be the enzyme that catalyzes conversion of NADAH to AcHA, and diamine oxidase to be responsible for the conversion of DAH to AmHA (30), our current studies provide even more information with regard to the biotransformation of HMBA and its metabolites. The time course of cellular accumulation of HMBA and each metabolite is compatible with the initial deacetylation of HMBA to NADAH and subsequent oxidation and deacetylation reactions that produce AcHA, DAH, and AmHA. Furthermore, the metabolic profiles found in cells incubated with various HMBA metabolites show the unidirectional, catabolic nature of these metabolic pathways. In no case was there evidence of a more proximal metabolic species such as HMBA or NADAH being present in cells incubated with a more distal metabolic form such as DAH, AcHA, or AmHA. The results of these particular studies also suggest that AmHA is derived primarily from DAH with a relatively minor contribution via deacetylation of AcHA. This implies different deacetylases being involved in the sequential conversion of HMBA to NADAH and DAH than are involved in deacetylation of AcHA to AmHA. This latter process may well involve an enzyme similar, if not identical, to N-acetyl-β-alanine deacetylase (39). This enzyme is different from the more common α-amino acid deacetylases and is known to have decreased ability to deacetylate amino acids, such as N-acetyl-γ-amino butyric acid, in which the acetamido moiety is separated from the carboxylic acid functionality by more than 1 methylene unit.

It is consideration of the sequential deacetylation of HMBA to NADAH and DAH that may provide the most interesting aspects of the current studies. One enzyme likely to carry out these biotransformations is N²-acetylserpinamide deacetylase (40), an enzyme known to convert NADAH to DAH (41). This particular enzyme, which converts N²-acetylserpinamide to spermidine, is known to be involved in regulation of ornithine decarboxylase activity (41-43). Furthermore, unlike polyamine oxidase, a cytosolic enzyme involved in polyamine recycling through conversion of N²-acetylserpinamide to putrescine, N²-acetylserpinamide deacetylase metabolizes a substrate produced by nuclear acetylases which are also involved in acetylation of histones (44-46). The mechanistic implications of involvement of a compound known to produce cellular differentiation with such enzyme systems are intriguing. This theory becomes even more plausible when considered in light of our demonstration that, of HMBA and its 4 documented metabolites, the only two with the ability to induce cellular differentiation are substrates for this particular enzyme. The concept that the mechanism of action of HMBA is related to its biotransformation by N²-acetylserpinamide deacetylase is made even more attractive by our observation that MELR cells can take up HMBA but contain low concentrations of NADAH and no DAH. This implies a decreased activity of the proposed target enzyme system in these resistant cells. At present we are actively pursuing each of these hypotheses further in hopes of shedding more light on the mechanism of action of HMBA and of allowing its more intelligent use in a clinical setting.

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