

# Identification of Metabolites of the Cell-differentiating Agent Hexamethylene Bisacetamide in Humans<sup>1</sup>

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## ABSTRACT

Hexamethylene bisacetamide, a compound which *in vitro* induces differentiation in a wide variety of human and animal cancer cell lines, is being investigated in phase I clinical trials. After i.v. administration of hexamethylene bisacetamide to humans, urine contained the parent compound and at least five metabolites formed by deacetylation and oxidation pathways. Identification of urinary metabolites was accomplished by gas chromatography-mass spectrometric analysis after isolation by ion exchange chromatography or extraction with ethyl acetate. Metabolites with amino or alcohol groups were trifluoroacetylated and acidic functional groups were esterified with 2,2,2-trifluoroethanol or methanol. The structure of each metabolite was confirmed by comparison with authentic standards. Metabolites identified included the major metabolite, 6-acetamidohexanoic acid; the monodeacetylated product, *N*-acetyl-1,6-diaminohexane; the bis-deacetylated diamine, 1,6-diaminohexane; and the amino acid, 6-aminohexanoic acid and its lactam, caprolactam.

## INTRODUCTION

HMBA<sup>3</sup> (NSC-95580) is a compound known to induce *in vitro* morphological and functional differentiation of murine and human leukemic cells (1-6) as well as a variety of human and solid tumor cell lines (7-11). Among the class of agents which have the potential of inducing the differentiation of tumor cells and which represent an exciting and novel approach to the chemotherapy of neoplasia, HMBA has a number of characteristics which render it one of the most interesting and of greatest potential clinical use. HMBA was selected for introduction into clinical trials because in the series of bisacetamides, HMBA, which has six methylene units separating the acetamide moieties, approached maximum differentiation potency (1, 3). In addition, HMBA differs from previous differentiating agents such as dimethyl sulfoxide and *N*-methylformamide that have undergone clinical evaluation in that carefully conducted clinical and pharmacokinetic studies have documented the ability to achieve concentrations of HMBA in patient plasma equal to the concentrations required for induction of differentiation *in vitro*.<sup>4</sup> On the other hand, administration of HMBA to humans has not proven devoid of adverse effects.<sup>4</sup> At HMBA dosages equal to or greater than 33 g/m<sup>2</sup>/day, systemic acidosis and neurotoxicity occur as dose limiting toxicities. Platelet count suppression, although not dose limiting, has also been observed with HMBA therapy. In view of the fact that the metabolism of HMBA was undefined and with the belief that knowledge of

the metabolic pathways of the drug might allow a better understanding of the mechanism of action and/or etiology of the toxicities associated with its use, we undertook the identification of HMBA metabolites found in humans treated with the drug. In this communication we report the structure elucidation of five metabolites of HMBA in the urine of patients treated with HMBA in a phase I trial at our institution.

## MATERIALS AND METHODS

**Chemicals and Instrumentation.** Hexamethylene bisacetamide was supplied by the Investigational Drug Branch, National Cancer Institute, Bethesda, MD. 1,6-Diaminohexane, 6-acetamidohexanoic acid, 6-aminohexanoic acid, 6-amino-1-hexanol, caprolactam, acetic anhydride, trifluoroacetic anhydride, and 2,2,2-trifluoroethanol were obtained from Aldrich Chemical Co. (Milwaukee, WI). 6-Acetamido-1-hexanol was *N*-acetylated with acetic anhydride in glacial acetic acid cooled in an ice bath. After stirring for 20 h at room temperature, the mixture was reduced in volume with a rotary evaporator to remove volatile solvents. Analysis of the product by GC-MS directly and as the ester derivative, 6-acetamido-1-hexyl trifluoroacetate, indicated that the major component was 6-acetamido-1-hexanol.

Proton nuclear magnetic resonance spectra were recorded on a Varian T-60A spectrometer and are expressed in ppm relative to internal standard 3-(trimethylsilyl)-1-propanesulfonic acid sodium salt.

**Synthesis of 6-Acetamido-1-aminohexane Hydrochloride.** Following a modified method of Tabor *et al.* (12), 1,6-diaminohexane (38.3 g; 330 mmol) was added slowly to glacial acetic acid (250 ml) which was stirred and cooled in an ice bath. The solution was heated to 70-80°C and acetic anhydride (9.0 ml; 95 mmol) was added dropwise with stirring over a 20-min period. The resulting mixture was stirred at room temperature for 4 h and then evaporated to dryness with a rotary evaporator. The residue was dissolved in 150 ml of hot water and concentrated HCl was added until pH 1 was achieved. The solution was evaporated to dryness and the residue was extracted with hot isopropyl alcohol to separate the product from the less isopropyl alcohol-soluble dihydrochloride of unreacted starting material. Repeated recrystallizations of the product from isopropyl alcohol/ether yielded pure material: mp 136-137°C; <sup>1</sup>H nuclear magnetic resonance (D<sub>2</sub>O) 1.17-1.90 (m, 6 H, CH<sub>2</sub>), 1.95 (s, 3 H, CH<sub>3</sub>), and 2.73-3.40 ppm (m, 4 H, CH<sub>2</sub>N).

**Drug Administration and Isolation of HMBA Metabolites.** HMBA was administered to patients as a 5-day continuous infusion at dosages of 4.8-43.2 g/m<sup>2</sup>/day. Urine was collected as voided and pooled as 24-h collections held at 4°C. The volume of each 24-h collection was determined daily and a portion was immediately stored at -20°C until analyzed.

The urine was thawed at room temperature, centrifuged at 2000 × g for 5 min, and 0.5-ml aliquots were taken for metabolite isolation. At this point, the urine aliquots were either evaporated to dryness with a rotary evaporator or separated into acid, base, and neutral fractions by ion exchange chromatography. Caprolactam was isolated by extraction of a 17-ml urine sample with two 25-ml portions of ethyl acetate. The combined ethyl acetate layers were dried (Na<sub>2</sub>SO<sub>4</sub>) and evaporated on a rotary evaporator.

Metabolite isolation was accomplished with cation and anion exchange resins. After acidification with 1 N HCl (0.5 ml), the urine was added to a column containing 5 g of AGW-50X8, 50-100 mesh cation exchange resin. The column was washed with water until the eluent was at neutral pH and then eluted with 1 N NH<sub>4</sub>OH (30 ml). The water

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<sup>3</sup> The abbreviations used are: HMBA, hexamethylene bisacetamide; GC, gas chromatography; MS, mass spectrometry; EI, electron ionization; CI, chemical ionization.

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wash contained neutral and acidic compounds whereas the  $\text{NH}_4\text{OH}$  fraction consisted of basic and amphiprotic metabolites. The water wash of the AGW-50 column was alkalinized with 1 N NaOH and then applied to a column containing 5 g of AGW-1X8, 50–100 mesh anion exchange resin. Elution with 1 N HCl (30 ml) gave a fraction which contained acidic metabolites. The acid, base, and neutral fractions were evaporated to dryness with a rotary evaporator.

**Derivatization of Metabolites.** Formation of trifluoroacetyl derivatives of amino and alcohol functional groups was accomplished by dissolution in trifluoroacetic anhydride (0.2 ml) in tubes sealed with Teflon-lined caps. Esters of acidic groups were formed by addition of 2,2,2-trifluoroethanol or methanol (0.1 ml) to the tubes which were then heated for 30 min at 50–60°C. Volatile solvents were removed from the resulting solutions with a stream of nitrogen and the residue was dissolved in ethyl acetate for GC-MS analysis.

**Simultaneous Gas Chromatography-Electron Ionization-Chemical Ionization-Mass Spectrometry.** A dual-source mass spectrometer was used to obtain both EI and CI spectra during a single chromatographic run (13). GC was carried out on a Carlo Erba 4130 instrument equipped with a 15-m Durabond 1701 capillary column with helium as carrier gas. After a 1-min solvent delay, the column was heated from a starting temperature of 50–250°C at 15°C/min. The column was directly interfaced with an Extrel Simulscan mass spectrometer. Electron ionization spectra were obtained with an ionizing voltage of 70 eV. Conditions for CI were approximately 1 torr source pressure of methane reagent gas with an ionizing voltage of 300 eV. Data acquisition was under the control of an Extrel 1000 data system. Alternate EI and CI spectra were acquired at a scan rate of 500 atomic mass units/s over a mass range of 60–500. At least 10 scans were recorded for each unknown GC peak. Data reduction involved summation or averaging of spectra and background subtraction to obtain representative spectra of unknowns.

As a control for potential artifactual interconversion of parent compound and metabolites during urine sample work-up and derivatization, mass spectral analyses were carried out on urine aliquots spiked with each metabolite.

## RESULTS

**Identification of HMBA Metabolites.** Following continuous i.v. infusions of HMBA in humans, 24-h urine collections were analyzed by GC-MS. Metabolite isolation was based on ion exchange chromatography or extraction with ethyl acetate. In order to facilitate GC analysis, a derivatization scheme was used that converted polar functional groups present in metabolites to less polar, more volatile derivatives. By reaction of urine extracts with trifluoroacetic anhydride and 2,2,2-trifluoroethanol, amino and alcohol groups were trifluoroacetylated and acidic functionalities were esterified. Fig. 1 shows a typical simultaneous EI/CI chromatographic run where relative peak intensities are represented by computer reconstructed summation of total ion currents derived from each mass spectrometer scan. EI and CI spectra were obtained from alternate scans. The utility of the CI spectra was in establishing the molecular weight of the derivatized metabolites by detection of protonated molecular species ( $\text{MH}^+$ ) and reagent gas cluster ( $\text{M}+29$ )<sup>+</sup> ions. Whereas the EI spectra for the most part gave weak molecular ions, analysis of fragmentation patterns and comparison of metabolite spectra to spectra of authentic standards provided strong evidence in support of assigned structures. In Table 1 are listed structures of HMBA metabolites and of derivatized HMBA metabolites. Table 2 lists the EI spectra of derivatized metabolites along with proposed structures for the major fragments. CI spectra with assignments for major fragment ions consistent with proposed structures are compiled in Table 3.

A major component excreted in urine, which was detected with a longer retention time than that of the derivatized metab-

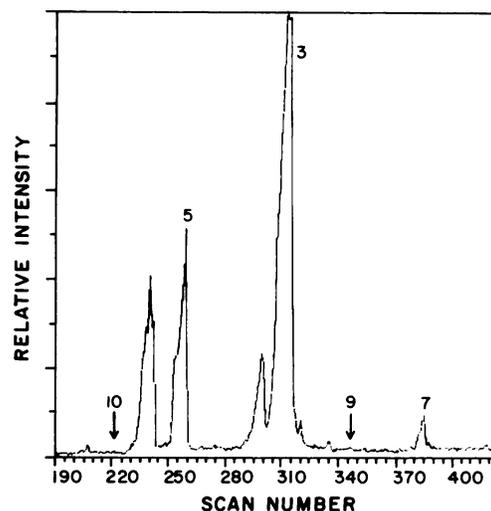


Fig. 1. Total ion current recording of a typical GC-MS chromatographic run of derivatized HMBA metabolites. Numbers within fig. correspond to structures in Table 1.

Table 1 Structures of HMBA metabolites and of derivatives of HMBA metabolites

RNH(CH <sub>2</sub> ) <sub>5</sub> R'					
Metabolites			Derivatized metabolites		
Compound	R	R'	Compound	R	R'
2	CH <sub>3</sub> CO	COOH	3	CH <sub>3</sub> CO	COOCH <sub>2</sub> CF <sub>3</sub>
4	H	COOH	5	CF <sub>3</sub> CO	COOCH <sub>2</sub> CF <sub>3</sub>
6	CH <sub>3</sub> CO	CH <sub>2</sub> NH <sub>2</sub>	7	CH <sub>3</sub> CO	CH <sub>2</sub> NHCOCF <sub>3</sub>
8	H	CH <sub>2</sub> NH <sub>2</sub>	9	CF <sub>3</sub> CO	CH <sub>2</sub> NHCOCF <sub>3</sub>
10	Caprolactam				

Table 2 Occurrence, relative abundance, and proposed structures of major ions in the electron ionization mass spectra of HMBA and derivatized HMBA metabolites

m/z <sup>a</sup>	Structure assignment <sup>b</sup>	Relative abundance (%) of Compound				
		1	3	5	7	9
69	(CF <sub>3</sub> ) <sup>+</sup> c		32	91	54	100
72	(AcNHCH <sub>2</sub> ) <sup>+</sup>	83	100		100	
83	(CF <sub>3</sub> CH <sub>2</sub> ) <sup>+</sup>		23	28		
86	(AcNHC <sub>2</sub> H <sub>4</sub> ) <sup>+</sup>	79	39		53	
100	(AcNHC <sub>3</sub> H <sub>6</sub> ) <sup>+</sup>	49	32		44	
114	(AcNHC <sub>4</sub> H <sub>8</sub> ) <sup>+</sup>	30	83		22	
126	(CF <sub>3</sub> CONHCH <sub>2</sub> ) <sup>+</sup>			100	14	47
128	(C <sub>3</sub> H <sub>5</sub> NOF <sub>3</sub> ) <sup>+</sup>				31	83
128	(M-AcNHCH <sub>2</sub> ) <sup>+</sup>	100				
140	(CF <sub>3</sub> CONHC <sub>2</sub> H <sub>4</sub> ) <sup>+</sup>		7	46	13	
140	(M-C <sub>2</sub> H <sub>4</sub> NO) <sup>+</sup>	27				
155	(CF <sub>3</sub> CH <sub>2</sub> OOC <sub>2</sub> H <sub>4</sub> ) <sup>+</sup>		7	23		
M-69	(M-CF <sub>3</sub> ) <sup>+</sup>			5	7	
M-43	(M-CH <sub>3</sub> CO) <sup>+</sup>	12	7			

<sup>a</sup> Ac, acetyl.

<sup>b</sup> Molecular ions were detected for all compounds as follows: Compound 1, m/z 200 (6); Compound 3, m/z 255 (15); Compound 5, m/z 309; Compound 7, m/z 254 (6); Compound 9, m/z 308; Compound 10, m/z 113 (100); the numbers in parentheses are the percent relative intensities for the ions. Other significant ions: Compound 1, m/z 185 (M-CH<sub>3</sub>); Compound 5, m/z 210 (M-CF<sub>3</sub>CH<sub>2</sub>O), m/z 68 (C<sub>5</sub>H<sub>8</sub>), m/z 96 (C<sub>6</sub>H<sub>8</sub>O), m/z 165; Compound 7, m/z 83; Compound 9, m/z 82 (C<sub>6</sub>H<sub>10</sub>), m/z 100, m/z 114 (C<sub>2</sub>H<sub>5</sub>NOF<sub>3</sub>)<sup>+</sup>; Compound 10, m/z 85 (M-CO or M-C<sub>2</sub>H<sub>4</sub>), m/z 84 (M-CHO or M-C<sub>2</sub>H<sub>5</sub>), m/z 67 (M-C<sub>2</sub>H<sub>3</sub>NO).

<sup>c</sup> Proposed structure of fragment ions.

olites, was identified as unchanged HMBA (Compound 1). The EI and CI spectra of the HMBA recovered from urine were the same as the spectra obtained for authentic HMBA.

The structure of the major metabolite isolated from urine was deduced to be 6-acetamidohexanoic acid (Compound 2). Esterification with 2,2,2-trifluoroethanol produced the derivative, 2,2,2-trifluoroethyl 6-acetamidohexanoate (Compound 3) which had the same gas chromatographic and mass spectrometric properties as authentic 6-acetamidohexanoic acid ester-



of action studies and by allowing a better understanding of the toxicities associated with use of the drug.

Although the ability of HMBA to induce differentiation has been demonstrated, the question of how HMBA metabolites influence differentiation remains an undefined area. Such an influence might occur in a number of ways. It remains unknown whether tumor cells that can be induced to differentiate do metabolize HMBA. Even if tumor cell metabolism of HMBA is not related to differentiation, the influence of metabolites, which might be generated in the liver or other site and be present in plasma, on HMBA induced differentiation is an important issue. If any of the metabolites of HMBA have their own differentiating potential or could enhance that of HMBA, the achievement and maintenance of 2 mM concentrations of HMBA in plasma may not be necessary; rather, a total of combined 2 mM of "differentiating material," consisting of HMBA plus metabolites, may be sufficient. This would have obvious practical importance in terms of the amount of drug required for administration. Similarly, since the toxicities associated with the use of HMBA are dose related, the administration of less drug would reduce the likelihood of evoking acidosis or neurotoxicity. Alternatively, the possibility exists that one or more of the metabolites of HMBA might inhibit the differentiating ability of HMBA. If tumor cells do not metabolize HMBA extensively, *in vitro* studies with HMBA alone might be flawed and similar studies should be done with mixtures of HMBA with the appropriate concentrations of those metabolites found in plasma of patients receiving HMBA. Studies addressing each of these issues are currently underway in our laboratories.

Knowledge of the biotransformation of HMBA may have more immediate implications in understanding the toxicities associated with use of the drug. The anion gap metabolic acidosis observed with administration of large doses of HMBA may likely reflect the associated production of 6-acetamidohexanoic acid, a material present in urine at concentrations approximately equal to those of HMBA. Since about 40% of a daily dose of HMBA is excreted unchanged in urine,<sup>4</sup> the maximum tolerated dose of approximately 30 g/m<sup>2</sup>/day would produce about 20 g of parent compound and therefore 20 g of 6-acetamidohexanoic acid in urine. Generation of 20 g/day of this organic acid may well be the source of the acidosis produced; if so, definition of the exact enzymatic steps leading to production of 6-acetamidohexanoic acid may allow development of strategies to prevent its production or maneuvers, as simple as coadministration of sodium bicarbonate, to affect the acid load generated from HMBA. Such strategies could greatly enhance the therapeutic index of HMBA. The central nervous system toxicities attributed to HMBA may be to some extent related to the acidosis produced. Alternatively, toxicities might be ascribed to generation of the various mono- and diamines found in the urine of patients receiving the drug; again, development of rational strategies to prevent production of either acidosis or specific metabolites of HMBA may allow more optimal use of the drug.

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