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SUBSTRATE SPECIFICITY OF BLEOMYCIN HYDROLASE*

SAID M. SEBTI,†‡§ JOHN C. DELEON,† LING-TAI MA,‖ SIDNEY M. HECHT‖ and JOHN S. LAZO†‡

† Department of Pharmacology, Yale University, School of Medicine, New Haven, CT 06510; and
‖ Departments of Chemistry and Biology, University of Virginia, Charlottesville, VA 22901, U.S.A.

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Abstract—Bleomycin (BLM) hydrolase is believed to protect both malignant and normal tissue from the toxicity of the antitumor drug BLM. Little is known about the substrate specificity of BLM hydrolase. Thus, we developed ion-paired reverse phase high speed liquid chromatography systems to assay for the metabolism of several BLM analogs. We found that BLM A_2, BLM B_2, tallysomycin S_{10b} (TLM S_{10b}), peplomycin (PEP), butylamino-3-propylamino-3-propylamine bleomycin (BAPP), deglyco bleomycin A_2 (dgBLM A_2) and bleomycinic acid were each metabolized by rabbit lung BLM hydrolase to a single metabolite. When compared to their corresponding parent compounds, these metabolites were 6- to 35-fold less potent in their ability to inhibit the proliferation of A-253 human head and neck squamous carcinoma cells in culture. Furthermore, we found that substitutions in various regions of the BLM molecule greatly affected the kinetic parameters of BLM hydrolase. For example, the K_m with BLM B_2 (0.056 ± 0.005 mM) was 15-fold lower than that seen with BLM A_2 (0.83 ± 0.11 mM). In contrast, the V_{max} was not affected markedly by these terminal amine substitutions but was influenced greatly by deletion of the carbohydrate groups of BLM. For example, a 4-fold higher V_{max} was observed with dgBLM A_2 compared to BLM A_2. Thus, these results demonstrate that BLM hydrolase can recognize and metabolize a broad spectrum of BLM analogs regardless of their structural features. This enzymatic conversion resulted in the inactivation of the BLMs as demonstrated by a substantial decrease in their cytotoxicity. Furthermore, the terminal amine and carbohydrate regions, respectively, dictate the apparent affinity and the rate of metabolism of BLM hydrolase substrates.

The bleomycins (BLMs¶), a family of glycopeptides isolated from *Streptomyces verticillus*, are widely used to treat human malignancies [1]. One unique feature of this class of antitumor agents is the lack of hepatic, myelosuppressive and renal toxicities [1, 2]. Pulmonary fibrosis, however, limits the usefulness of the current clinical mixtures of BLMs [1, 2]. Studies with BLM A_2 and BLM B_2, which represent about 70 and 30% of this clinical mixture, respectively, suggest that *in situ* metabolism of these analogs regulates their antitumor activity and organ toxicity [1, 2]. It is believed that BLM hydrolase, which hydrolyzes the amide bond in the beta-aminoalanine moiety of the BLM molecule, plays an important role in the resistance of some tumors to BLM [3–5] and in the protection of lungs and other normal tissues from BLM-induced toxicity [2, 6, 7]. Deamido BLM A_2 and deamido BLM B_2, the metabolites formed following the hydrolysis of BLM A_2 and BLM B_2 by BLM hydrolase, are markedly less toxic than their parent compounds [2, 6, 7].

The biochemical characteristics of BLM hydrolase have been examined only recently in detail [8–10]. Sebti *et al.* [8] purified BLM hydrolase to homogeneity from rabbit lungs. The labile enzyme has a pH optimum of 7.0 to 7.5 and exhibits properties of a thiol-containing 250 kD pentamer with 50 kD subunits. The enzyme is strongly inhibited by *N*-ethylmaleimide, leupeptin, puromycin and divalent cations [8, 9]. Rabbit liver BLM hydrolase was also purified independently to homogeneity using an anti-BLM hydrolase monoclonal antibody and was found to have biochemical properties and inhibitor profiles similar to the lung enzyme [10]. The substrate specificity of BLM hydrolase, however, has not been examined in any detail. Studies with BLM A_2, BLM B_2 and PEP showed that these analogs with different terminal amines are metabolized to their corresponding deamido metabolites [2, 3, 10]. Preliminary kinetic studies by Nishimura *et al.* [10] with purified rabbit liver BLM hydrolase indicate that the V_{max} seen with BLM B_2 differs from that seen with PEP. Lacking, however, has been a systematic kinetic examination of BLM analogs with structural changes in different chemical domains of the BLM molecule. The purpose of our studies was to use existing BLM analogs to: (a) determine whether or not these analogs are metabolized by BLM hydrolase; (b) map the regions of the BLM molecule that affect the kinetic parameters (K_m and V_{max}) of BLM

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‡ Present address: Department of Pharmacology, University of Pittsburgh, School of Medicine, 518 Scaife Hall, Pittsburgh, PA 15261.

§ Send reprint requests to: Dr. Said M. Sebti, Department of Pharmacology, University of Pittsburgh, School of Medicine, 518 Scaife Hall, Pittsburgh, PA 15261.

¶ Abbreviations: BLM, bleomycin; TLM, tallysomycin; PEP, peplomycin; BAPP, butylamino-3-propylamino-3-propylamine bleomycin; dgBLM A_2, deglycobleomycin A_2; HSLC, high speed liquid chromatography.

hydrolase and (c) determine whether or not metabolism of the BLMs by BLM hydrolase affects their antitumor activities against cultured human tumor cells.

MATERIALS AND METHODS

Bleomycin analogs. BLM A_2 and BLM B_2 were purified from Blenoxane (Bristol Laboratories, Wallingford, CT) as described previously [11]. PEP and BAPP were gifts from the Development Therapeutics Program of the National Cancer Institute. TLM S_{10b} was obtained from Dr. W. T. Bradner (Bristol Laboratories). Bleomycinic acid and dgBLM A_2 were synthesized as described by Umezawa *et al.* [12] and Sugiyama *et al.* [13] respectively. Authentic BLM dA_2 and BLM dB_2 standards were provided by Dr. A. Fujii (Nippon Kayaku, Ltd., Japan) and additional BLM dA_2 and BLM dB_2 were enzymatically prepared as described previously [6]. Each compound was metal free and was over 95% pure as judged by reverse phase HSLC as described below.

Purification of BLM hydrolase. BLM hydrolase was purified from the lungs of albino New Zealand rabbits as described by Sebti *et al.* [8]. Briefly, the lungs were homogenized and centrifuged at 20,000 *g* and 105,000 *g*. The 105,000 *g* supernatant fractions were then chromatographed on a DEAE-Sephacel column, and those fractions with BLM hydrolase activity were purified by chromatography on a phenyl-Sepharose column [8]. The resulting BLM hydrolase preparation was 1000-fold purified and was used for all the studies described in this paper unless specified otherwise in the text. To confirm the results carried out with the 1000-fold purified preparation, we purified BLM hydrolase to homogeneity (6000-fold) as described previously [8] and used it with BLM A_2 and BLM B_2 as substrates. Only BLM A_2 and BLM B_2 were used in these confirmatory studies due to the limited amounts of BLM hydrolase purified to homogeneity.

Separation of the various BLM analogs from their corresponding metabolites by HSLC. BLM A_2, BLM B_2, TLM S_{10b}, PEP, bleomycinic acid, dgBLM A_2, and BAPP (0.2 to 1 mM) were incubated with (native or denatured) purified rabbit pulmonary BLM hydrolase (27 μg protein/ml) in the presence of 10 mM Tris–HCl (pH 7.5) for various periods of time. The reaction was stopped by adding an equal volume of methanol, vortexing, and centrifuging. Aliquots of the supernatant fractions were analyzed by reverse-phase HSLC, a narrow-bore column (3 μm particle size) analytical system described by Sebti and Lazo [9]. The mobile phases used to optimize the resolution between each BLM analog and its corresponding metabolite are shown in Table 1. All BLM analogs and their metabolites were detected fluorometrically using an excitation wavelength of 292 nm (10 nm band pass) and an emission wavelength of 355 nm (10 nm band pass) [9, 11].

Kinetic studies. All BLM analogs were incubated with 1000-fold purified BLM hydrolase at a concentration of 27 μg protein/ml in the presence of 10 mM Tris–HCl (pH 7.5) at 37°. BLM A_2 and BLM B_2 were also incubated with the 6000-fold purified BLM hydrolase (2 μg/ml) under the same conditions. The concentration and time of incubation of each BLM analog were: BLM A_2 (0.2 to 2.8 mM and 15 min), BLM B_2 (0.04 to 2.1 mM and 15 min), TLM S_{10b}, (0.2 to 1.8 mM and 60 min), PEP (0.1 to 1.6 mM and 15 min), bleomycinic acid (0.05 to 6.5 mM and 20 min), dgBLM A_2 (0.2 to 3.0 mM and 15 min), and BAPP (0.2 to 2.0 mM and 45 min). All incubations were carried out under initial velocity conditions. The incubation mixtures were analyzed by HSLC as described above. For BLM A_2 and BLM B_2, the amounts of metabolites formed were calculated from the relative fluorescence of known amounts of the corresponding deamido standards. For the remaining analogs, the amounts of metabolites formed were calculated from the relative fluorescence of known amounts of parent compound standards analyzed by HSLC. The K_m (mM) and V_{max} (μmol of metabolite generated/mg·hr) were determined by standard Lineweaver–Burk analysis.

A-253 Cell growth inhibition assay. All BLM analogs were incubated for 14 hr at 37° in the presence or absence of the purified BLM hydrolase preparation (270 μg protein/ml) in 10 mM Tris–HCl (pH 7.5). The substrate concentration was 100 μM except for dgBLM A_2 (60 μM) and BAPP (50 μM). The reaction was stopped by decreasing the temperature to 4°, and aliquots were analyzed by HSLC for metabolite formation as described above. Aliquots of the reaction mixtures were also tested for their ability to inhibit the proliferation of human A-253 squamous carcinoma cells [14] using the method of Finlay *et al.* [15]. Briefly, cells obtained from exponentially growing monolayer cultures were placed in 96-well microtiter plates at a density of 4×10^3 cells/cm^2.

Table 1. Mobile phase compositions*

	H_2O* (%)	MeOH (%)	CH_3CN (%)	CH_3COOH (%)	Triethylamine (mM)	Heptane sulfonic acid (mM)
BLM A_2	79.5	12.5	7.2	0.8	25.0	2.0
dgBLM A_2	79.5	12.5	7.2	0.8	25.0	2.0
BLM B_2	73.2	19.5	6.6	0.7	2.0	1.8
BAPP	63.6	30.0	5.8	0.6	20.0	1.6
PEP	55.7	38.8	5.0	0.6	17.5	1.4
TLM S_{10b}	82.6	10.6	6.1	0.7	21.3	1.7
Bleomycinic acid	84.6	9.4	5.4	0.6	18.8	1.5

* The pH of all mobile phases was 5.5.

Various volumes of the reaction mixture were added to the medium, and the cells were incubated for 4 days at which time the medium was removed, and the cells were stained with 0.5% (w/v) methylene blue and solubilized with 1% Sarkosyl in phosphate-buffered 0.9% NaCl. Twenty-four hours later, the cell density for each concentration of the BLM analog was determined spectrophotometrically (620 nm). The drug concentrations required to inhibit cell growth by 50% (IC_{50}) were then determined.

RESULTS

Metabolism of BLM analogs. Figure 1 shows the structures of the various BLM analogs used in this study. All of these analogs contain the bleomycinic acid structure, a glycopeptide that has a hydroxyl group as a terminal amine (see R_1 group in Fig. 1). PEP and BAPP contain hydrophobic terminal amines, whereas BLM A_2 and BLM B_2 contain relatively polar terminal amines. TLM S_{10b} contains an extra carbohydrate group (see R_2 group in Fig. 1), whereas dgBLM A_2, which is not shown in Fig. 1, corresponds to BLM A_2 without the two carbohydrate groups.

To determine whether or not BLM hydrolase metabolizes these various BLM analogs, we developed several chromatographic systems to separate the metabolites formed from their corresponding parent compounds. These chromatographic assays were adapted for each analog by a modification of our previously developed ion-paired reverse phase HSLC assay for the separation of BLM A_2 from its metabolite BLM dA_2 [9]. BLM A_2, BLM B_2, PEP, BAPP, TLM S_{10b}, dgBLM A_2 and bleomycin acid were incubated with rabbit lung BLM hydrolase for different lengths of time, and the reaction mixtures were analyzed by HSLC as described in Materials and Methods. The HSLC elution profiles of the BLM analogs and their corresponding metabolites after incubation of the parent compounds with native or denatured (heated) BLM hydrolase are shown in Fig. 2. When BLM hydrolase was boiled prior to incubation with the BLM analogs, only the parent compound eluted from the HSLC column (see chromatograms labeled "Heated" in Fig. 2). In contrast, when the analogs were incubated with native BLM hydrolase, two major peaks were eluted for each sample (Fig. 2). The larger peak coeluted with the parent compound, and the smaller peak (darkened area), which eluted earlier than the parent compound, was the BLM hydrolase generated metabolite. Similar results were also obtained when bleomycinic acid was used as the substrate (data not shown).

The metabolites were shown to be the products generated by BLM hydrolase and not by contaminants in the 1000-fold purified preparation by studying the metabolism of BLM A_2 and BLM B_2 with BLM hydrolase that was purified to homogeneity. The results were identical to those shown in Fig. 2 for BLM A_2 and BLM B_2. Similar experiments with the other BLM analogs were not carried out due to the limited amounts of the highly purified BLM hydrolase.

The metabolites formed from BLM A_2 and BLM B_2 were identified as BLM dA_2 and BLM dB_2, respectively, based on previous work [1, 4, 6, 7, 16,

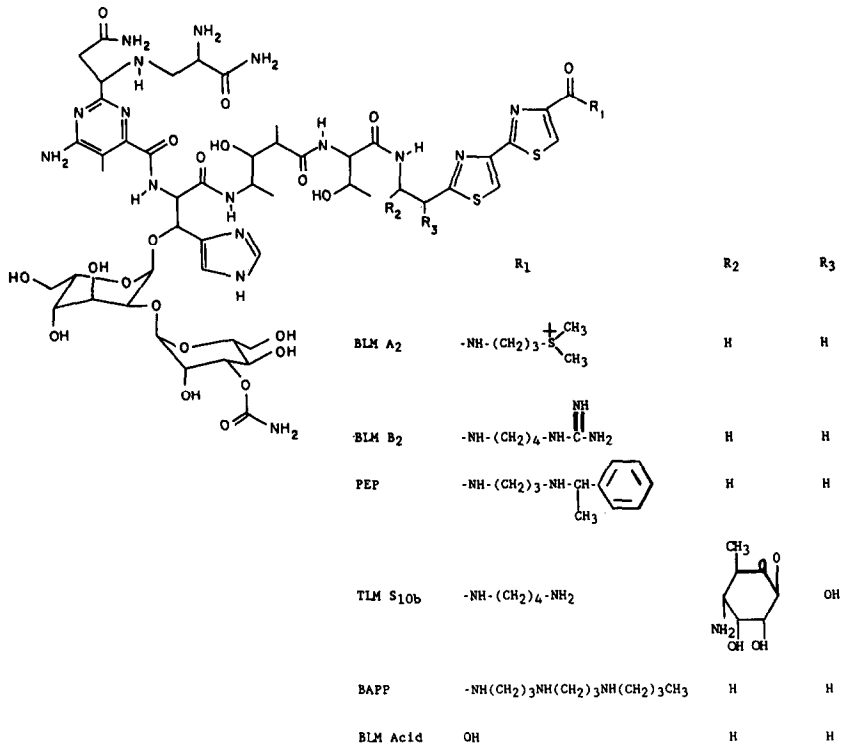

Fig. 1. Structures of BLM analogs.

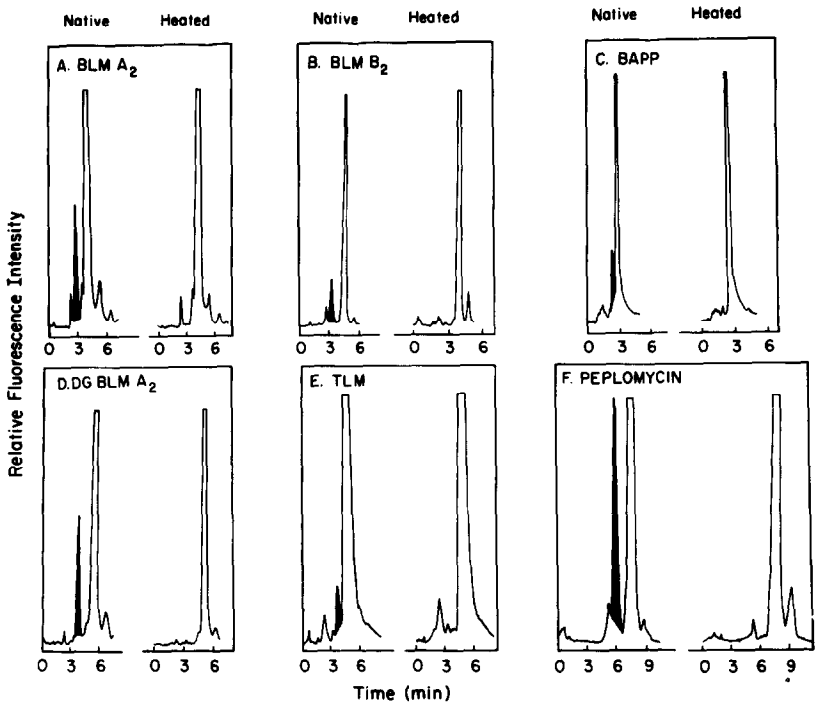

Fig. 2. HSLC profiles of the BLM analogs and their metabolites. BLM A_2 (panel A), BLM B_2 (panel B), BAPP (panel C), dgBLM A_2 (panel D), TLM S_{10b} (panel E), and PEP (panel F) were incubated with BLM hydrolase under native conditions (native) or BLM hydrolase that was previously denatured by boiling (heated). The reaction mixtures were then analyzed by HSLC as described in Materials and Methods. Darkened areas indicate the peak associated with the new metabolite.

17] and on coelution studies with BLM dA_2 and BLM dB_2 standards. Furthermore, results from mass spectrum analysis of the metabolites generated from BLM A_2 by BLM hydrolase are consistent with the formation of the deamination product, BLM dA_2 (data not shown). The metabolites formed from PEP, BAPP, TLM S_{10b}, dgBLM A_2 and bleomycinic acid were also tentatively identified as deamination products, based on previous work with PEP [10, 16] and based on their HSLC column elution position relative to their parent compounds. These profiles were very similar to those of BLM A_2/dA_2 and BLM B_2/dB_2 (Fig. 2). Thus, rabbit lung BLM hydrolase recognizes and metabolizes all the BLM analogs tested regardless of their structural features.

Relationship between metabolism of BLM analogs and their antiproliferative activity in cultured human tumor cells. To determine whether or not metabolism of the BLM analogs by BLM hydrolase affected their cytotoxicity, we incubated the analogs in the presence or absence of BLM hydrolase prior to testing their antiproliferative activity against A-253 human head and neck squamous carcinoma cells in culture. BLM A_2, BLM B_2, PEP, BAPP, dgBLM A_2, TLM S_{10b} and bleomycinic acid were incubated with BLM hydrolase for 14 hr, and aliquots of the reaction mixture were analyzed by HSLC as described in Materials and Methods. A 14-hr incubation period was chosen to maximize the relative proportion of metabolites formed. The HSLC elution profiles of these samples were similar to Fig. 2 except that over 90% of each parent drug was converted to the corresponding metabolite in samples incubated with BLM hydrolase, whereas in those samples incubated without BLM hydrolase metabolites were not detected and only the parent compounds were present (data not shown). Aliquots of these reaction mixtures were then incubated with A-253 cells and the ability of the parent compounds and their corresponding metabolites to inhibit the growth of these tumor cells was determined as described in Materials and Methods. A-253 cell survival curves of the BLM analogs with and without the BLM hydrolase pretreatment are shown in Fig. 3. Pretreatment of all of the BLM analogs with BLM hydrolase resulted in a substantial decrease in their antiproliferative activity relative to the activity of their intact parent compounds as indicated in Fig. 3. The concentrations (nM) of the BLM analogs that inhibited 50% of A-253 cell growth (IC_{50}) with or without BLM hydrolase pretreatment were, respectively, BLM A_2 (45 and 4.5 nM), BLM B_2 (48 and 1.3 nM), PEP (35 and 6 nM), TLM S_{10b} (38 and 5.8 nM), and BAPP (35 and 2.5 nM) (Fig. 3). The most prominent alteration in drug action was observed with BLM B_2, which was 37-fold less potent after incubation with BLM hydrolase, followed by BAPP (14-fold), BLM A_2 (10-fold), TLM S_{10b} (7-fold), and PEP (6-fold). Bleomycinin acid was not exceptionally potent (IC_{50} = 140 nM) and limited quantities of the parent compound prevented an accurate assessment of the IC_{50} of the bleomycinic acid metabolite. Nonetheless, incubation of bleomycinic acid with BLM hydrolase rendered it at least

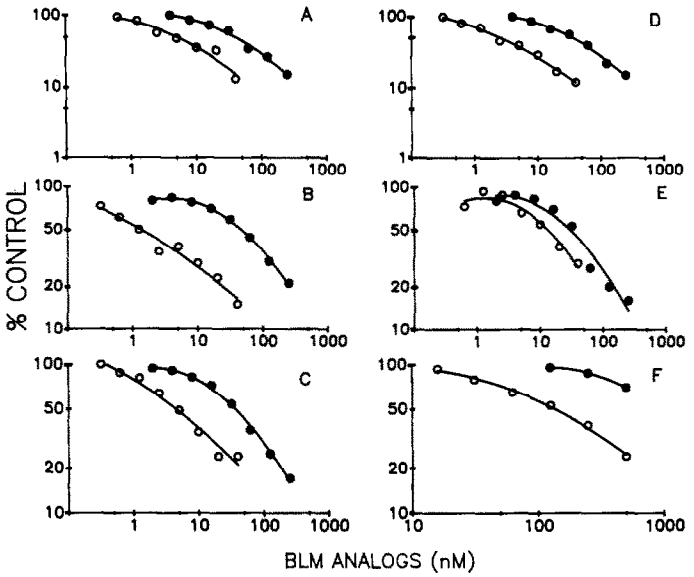

Fig. 3. Survival curves. Human head and neck A-253 squamous cell carcinoma cells were exposed to various concentrations of BLM analogs that had been incubated previously in the presence (●) or absence (○) of BLM hydrolase as described in Materials and Methods. Cellular growth was determined spectrophorometrically after staining with methylene blue [14, 15] as described in Materials and Methods. Key: (A) BLM A_2, (B) BLM B_2, (C) PEP, (D) BAPP, (E) TLM S_{10b}, and (F) bleomycinic acid.

7-fold less potent (Fig. 3). Similarly, small quantities and the low potency of dgBLM A_2 (IC_{50} = 1200 nM) limited our studies but loss of antiproliferative capability was observed (data not shown). Rabbit lung BLM hydrolase alone was not toxic to A-253 cells.

Kinetic parameters of BLM hydrolase substrates. To determine the regions in the BLM molecule that influence the interaction between the substrate and the enzyme and the rate of catalysis, we carried out kinetic studies under initial velocity conditions with each of the BLM analogs. Incubation of BLM hydrolase with various concentrations of BLM A_2, BLM B_2, PEP, TLM S_{10b}, BAPP and dgBLM A_2 resulted in maximum enzyme velocity, suggesting saturation of the enzyme. In contrast, no saturation of BLM hydrolase was observed with bleomycinic acid even at concentrations as high as 6.5 mM. Limited quantities of bleomycinic acid restricted the use of greater concentrations of drug. Figure 4 shows representative Lineweaver–Burk plots of BLM A_2 and BLM B_2 (panel A) and BLM A_2 and dgBLM A_2 (panel B). BLM B_2, which has an agmatine as a terminal amine (Fig. 1), had a K_m 15-fold lower than BLM A_2, which has a dimethyl sulfonium propylamine as the terminal amine (Fig. 4, panel A). BLM A_2 and dgBLM A_2 had similar K_m values but there was approximately a 4-fold difference in their V_{max} values (Fig. 4, panel B). The K_m and V_{max} for BLM A_2 and BLM B_2 were confirmed by using BLM hydrolase purified to homogeneity (6000-fold) [8], indicating that the small amounts of contaminants in the 1000-fold purified BLM hydrolase preparation used in these studies did not affect the K_m and V_{max} values. We extended the kinetic analysis to include additional BLM analogs. As seen in Fig. 5, the mean result of four or more independent determinations

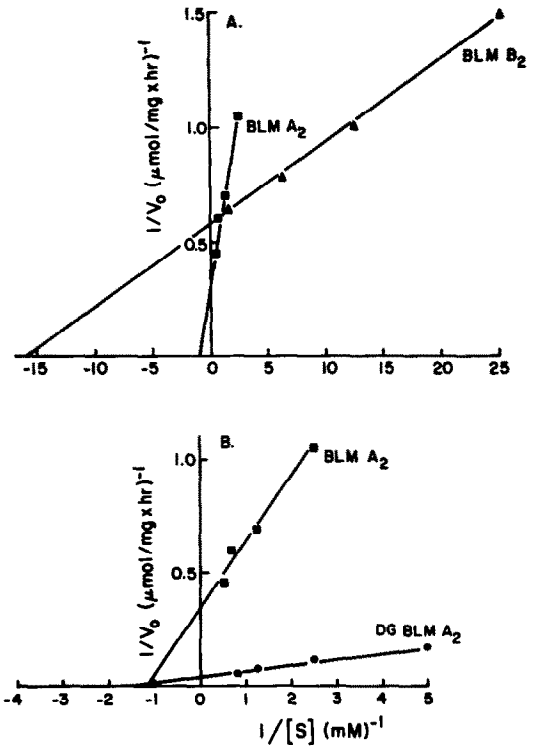

Fig. 4. Lineweaver–Burk plots of some BLM hydrolase substrates. Various concentrations of BLM A_2 and BLM B_2 (panel A) and BLM A_2 and dgBLM A_2 (panel B) were incubated with BLM hydrolase and analyzed by HSLC as described in Materials and Methods. The velocity (V_0) was then determined for each BLM analog and the $1/V_0$ was plotted vs $1/[S]$. $[S]$ designates the BLM analog concentration.

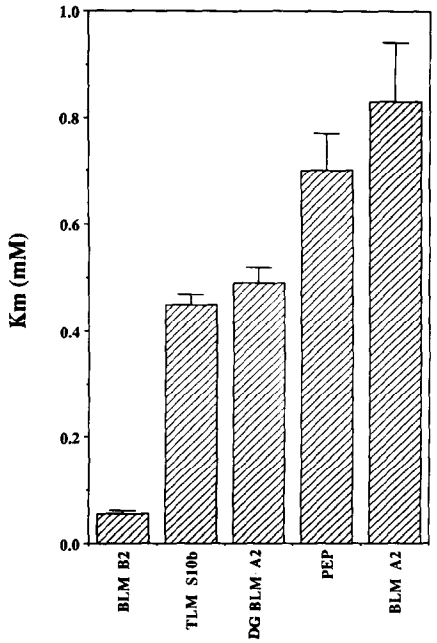

Fig. 5. K_m values of various BLM hydrolase substrates. The BLM analogs were incubated with BLM hydrolase and analyzed by HSLC as described in Materials and Methods. The K_m values were then determined by Lineweaver–Burk analysis as described in the legend of Fig. 4. Each kinetic experiment was repeated at least four times.

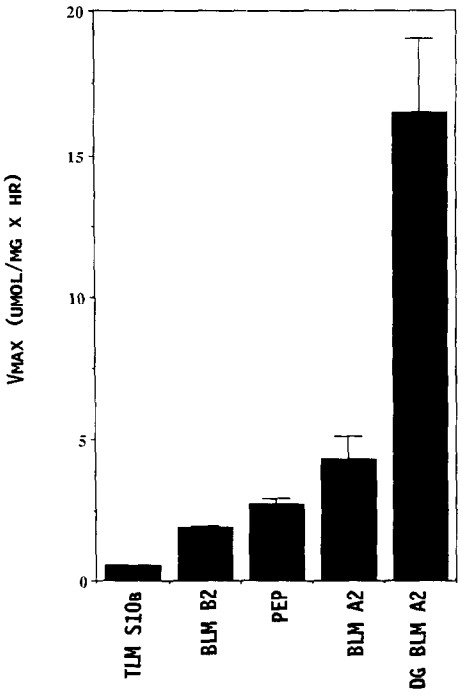

Fig. 6. V_{max} values of various BLM hydrolase substrates. The BLM analogs were incubated with hydrolase and analyzed by HSLC as described in Materials and Methods. The V_{max} values were then determined by Lineweaver–Burk analysis as described in the legend of Fig. 4. Each kinetic experiment was repeated at least four times.

indicates that BLM B_2 had the lowest K_m (0.056 ± 0.005 mM), followed by TLM S_{10b} (0.45 ± 0.02 mM), dgBLM A_2 (0.49 ± 0.03 mM), PEP (0.70 ± 0.07 mM), and BLM A_2 (0.83 ± 0.11 mM). The highest V_{max} of all analogs was observed with dgBLM A_2 (16.49 ± 2.56 μmol·hr), whereas TLM S_{10b} (0.540 ± 0.007 μmol/mg·hr) had the lowest and BLM B_2 (1.87 ± 0.05 μmol/mg·hr), PEP (2.73 ± 0.16 μmol/mg·hr) and BLM A_2 (4.3 ± 0.81 μmol/mg·hr) had intermediate V_{max} values (Fig. 6). BAPP, which was evaluated in a single experiment, had a K_m of 2.25 mM and a V_{max} of 8.71 μmol/mg·hr.

DISCUSSION

Although BLM is widely used to treat human malignancies, tumor cell resistance and BLM-induced pulmonary fibrosis limit its effectiveness. BLM analogs with improved activity and reduced pulmonary toxicity are therapeutically desirable, and several potential candidates, such as PEP and TLM S_{10b}, are now in clinical trials [16].

The role of BLM hydrolase in protecting both malignant and normal tissue from BLM-induced toxicity has been suggested by several investigators [1, 2, 4, 5]. Although significant progress has been made recently in purifying and characterizing BLM hydrolase [8–10], little is known about the substrate specificity of this enzyme. BLM has two major structural domains: the DNA binding region which includes the terminal amine and the bithiozole moiety, and the metal chelating region (Fig. 1) [1, 2]. Previous work by us and others showed that BLM hydrolase inactivates BLM B_2, BLM A_2 and PEP by hydrolyzing the amide bond of the beta alaninamide moiety of these molecules [1, 4, 6, 10, 16]. These analogs have identical metal binding regions but differ in their terminal amine (Fig. 1). It is not known, however, whether or not BLM hydrolase can recognize, metabolize and inactivate BLM analogs with alterations other than those at the terminal amine, nor have the structural features that affect the kinetic parameters of the BLM hydrolase reaction been determined. Our results demonstrate for the first time that a variety of structurally different BLM analogs were metabolized by BLM hydrolase. This enzyme metabolized BLM analogs with alterations in non-DNA binding domains (dgBLM A_2 and TLM S_{10b}) and analogs with terminal amines that are hydrophobic (PEP and BAPP), anionic (BLM B_2 and BLM A_2), or absent (bleomycinic acid). Furthermore, our studies with cultured human tumor cells allowed us to demonstrate that metabolism of all the BLM analogs tested resulted in a substantial decrease (6- to 37-fold) in their ability to inhibit the growth of A-253 tumor cells. The magnitude of this decrease in cytotoxicity was consistent with the difference in DNA damage caused *in vitro* by BLM A_2 and BLM dA_2 [18].

The nature of the terminal amine had dramatic effects on the K_m of BLM hydrolase. BLM B_2, which has an agmatine moiety at its terminal amine, had the lowest K_m of all the BLM analogs. BLM B_2 had a K_m 15-fold lower than the K_m of BLM A_2. In

contrast, substitution in other regions of the molecule, such as removal of the carbohydrate groups, did not greatly affect K_m values. The removal of these carbohydrate groups from BLM A_2, however, greatly increased maximal velocity of metabolism. These results clearly demonstrate that BLM hydrolase interacts with various regions of the BLM molecule and that these interactions were translated into major effects on the kinetic parameters of the reaction.

Thus, BLM hydrolase metabolized and inactivated all the BLM analogs tested in this study. Furthermore, the interaction of BLM hydrolase with the terminal amine region of BLM appears to be extremely important for its affinity toward the substrate, whereas the interaction of BLM hydrolase with the carbohydrate group region affects to a great extent the rate of metabolism of the BLM molecule. These results could be useful in future attempts to design new bleomycin analogs with altered anticancer activity or pulmonary toxicity.

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