Lidocaine Potentiation of Bleomycin A2 Cytotoxicity and DNA Strand Breakage in L1210 and Human A-253 Cells

John S. Lazo,1 I. Deborah Braun, Borislav Meandžija, Katherine A. Kennedy,2 Esther T. Pham, and Laurie F. Smaldone3


ABSTRACT

The survival of cultured L1210 cells exposed to bleomycin A2 (BLM A2) was markedly decreased by coincubation with the local anesthetic lidocaine. The potentiation occurred with concentrations of lidocaine that were nontoxic and was dependent upon both the concentration of lidocaine and BLM A2. A 1000-fold decrease in survival was seen with a 1-h exposure to 8 mM lidocaine and 10 mM BLM A2 compared to incubation with 10 mM BLM A2 alone. Prior exposure to lidocaine did not markedly alter BLM A2 cytotoxicity, while treatment with lidocaine immediately after BLM A2 exposure did, suggesting that increased cellular content of BLM A2 was not the mechanism of enhancement. Furthermore, lidocaine reduced the total amount of cell-associated radioactivity seen after incubation with [3H]BLM A2. The enhancement in L1210 cell cytotoxicity with lidocaine was not specific for the C- and N-terminal moieties of the BLM molecule. Other DNA-interacting antitumor agents, such as etoposide and mitomycin C, did not exhibit biologically significant alterations in their cytotoxicity when coincubated with lidocaine, although cis-diaminedichloroplatinum was significantly more toxic in the presence of lidocaine. The potentiation of BLM A2 cytotoxicity was not unique to murine tumor cells, since it was also seen with cultured human head and neck carcinoma (A-253) cells. Lidocaine did not increase directly BLM A2-induced breakage of DNA in vitro as measured by loss of form I PAT 153 DNA, but it did increase BLM A2-induced DNA strand breaks in intact L1210 cells coincubated with lidocaine and BLM A2. Exposure of L1210 cells to lidocaine after BLM A2 treatment also greatly increased DNA breakage consistent with possible inhibition of DNA repair. In addition, a modest reduction in the in vitro inactivation of BLM A2 by BLM hydrolase was found with lidocaine. We propose that inhibition of BLM metabolism and repair of BLM-induced DNA damage by lidocaine may have a role in the enhanced cytotoxicity.

INTRODUCTION

The bleomycins are glycopeptides, the antitumor activity of which is believed to result from DNA breakage (23). The clinical mixture comprises at least 13 bleomycins, with BLM A2 being the principal component. A variety of factors may influence the cytotoxicity of this class of antitumor antibiotics, and the two that are thought to be most important are cellular accumulation and metabolic inactivation of drug (23). Although the cellular pharmacokinetics of the bleomycins have only been poorly characterized, it appears that only small quantities of BLM enter cells, suggesting that entry of the drug may be an important regulatory process (19). Metabolism of the bleomycins to inactive species by an aminopeptidase B-like enzyme called bleomycin hydrolase may also influence the lethal action of these antitumor antibiotics. Murine cells with low levels of the inactivating activity have been reported to respond better to BLM than have cells with high levels (23).

Previous studies (1) have indicated that exposure of cells to agents such as filipin and pentamycin, which enhance the cellular content of BLM A2, increase BLM A2 cytotoxicity. More recently, Mizuno and Ishida (16, 17) reported that, in HeLa and FM3A cells, local anesthetics, such as lidocaine, enhance the cytotoxicity of the clinical bleomycin mixture, as well as a bleomycin derivative peplomycin. The structural requirements and the mechanisms for the observed potentiation are uncertain. Thus, we have characterized the cytotoxic interactions between lidocaine and several BLM analogues—in particular, the major component of the clinical mixture, BLM A2—using cultured murine L1210 and human head and neck squamous carcinoma cells (A-253). In addition, we have explored possible mechanisms for the enhanced cytotoxicity.

MATERIALS AND METHODS

Cell Culturing Techniques. L1210 murine leukemia cells were maintained in suspension culture with Fischer's medium and 10% horse serum, penicillin (100 units/ml) and streptomycin (100 μg/ml). Cells were routinely assayed and found free of Mycoplasma contamination. All studies were conducted with cells in exponential growth phase (2 to 3 x 10⁶ cells/ml). Cell numbers were determined with a Coulter model ZBI counter (Hialeah, FL). Single cell preparations of L1210 cells (5 x 10⁵ cells/ml) were exposed to various concentrations of copper-free BLM A2 (0.1 to 30 μM), BLM dA2 (1 to 50 μM), or other antitumor agents (1 or 2 μM) for 1 h at 37°C in the presence or absence of lidocaine (0.5 to 10 mM) and centrifuged at 200 x g for 5 min at 37°C. The medium was then removed, and the cell pellet was resuspended in 10 ml of Fischer's medium containing 15% horse serum. In other studies, BLM A2 was added 1, 1.5, or 2 h before or after adding 8 mM lidocaine. The cells were treated with the first drug for 1 h; the medium was decanted, and 2 ml of Fischer's medium with 10% horse serum containing or lacking the second drug was added. If drug-free medium was used, the second drug was added within 2 h of the first drug. In all cases, cells were incubated at 37°C for 1 h with the second drug. The drug containing medium was then decanted, and the cells were resuspended in drug-free medium. Survival was determined by the method of Chu and Fischer (4), in which between 100 and 10,000 cells were resuspended in a culture...
tube with Fischer's medium, 15% horse serum, and 0.13% agar and incubated for 10 to 12 days at 37°C in a 5% CO2, 95% air incubator. Colonies of approximately 250 μm or greater in diameter (approximately 8000 cells or 13 population doublings) were stained by adding 0.5 ml of a sterile solution (1 mg/ml) of 2-(p-iiodophenyl)-3-(p-nitrophenoxy)-5-phenyltetrazolium chloride and incubating at 37°C for 24 h. The colonies were then counted with a dissecting microscope (6.6x). The average cloning efficiency of untreated control cells for all experiments was 69.1 ± 3.6% (SE). The surviving fraction of cells was calculated from the ratio of the cloning efficiency of the experimental group to that of the untreated control. The A-253 human cells were obtained from the American Type Culture Collection (Rockville, MD) and were isolated originally from a human epidermoid carcinoma located in the submaxillary gland (5). A-253 cells were maintained as a monolayer in exponential growth phase with McCoy's 5a medium (modified) (Grand Island Biological Company, Grand Island, NY) containing 10% fetal bovine serum, penicillin (100 units/ml), streptomycin (100 μg/ml). They have a population doubling time of 24 h. exponentially growing cells were removed from a 150-cm2 plastic culture flask with a 1-min exposure to a phosphate-buffered NaCl solution (137 mM NaCl, 2.7 mM KCl, 8 mM NaHPO4, 1.5 mM KH2PO4, pH 7.4) containing 0.25% (v/v) trypsin (Grand Island Biological Company, Grand Island, NY) and 2 mM EDTA. after centrifugation at 200 × g for 4 min and washing with the phosphate-buffered NaCl solution, the cell concentration was determined with a Coulter counter, and the cells were resuspended in the growth medium containing serum (4 × 105 cells/ml) and were treated for 1 h with BLM A2 (0.7 μM) and lidocaine (8 mM). Cells were then washed free of drug and resuspended in drug-free McCoy's 5a medium (modified) containing 10% fetal bovine serum and 0.3% agar. The cells were quickly placed on top of a solidified solution of 0.5% agar containing McCoy's 5a medium and 10% fetal bovine serum. after 12 days in a 5% CO2, 95% air incubator (37°C), colonies were stained by adding 1 ml of solution containing 1 mg/ml solution of 2-(p-iiodophenyl)-3-(p-nitrophenoxy)-5-phenyltetrazolium chloride and incubating for 24 h at 37°C. Colonies with diameters greater than 80 μm were then counted with a dissecting microscope. The doubling time of cells in soft agar appeared to be longer than that in monolayer, and the average colony was between 80 and 140 μm in diameter. The average cloning efficiency of untreated control cells for all experiments was 69.1 ± 3.6% (SE). The surviving fraction of cells was calculated from the ratio of the cloning efficiency of the experimental group to that of the untreated control. Drugs. Copper-free BLM A2 was either isolated by our methods described previously (9) from Blenoxane (Bristol Laboratories, Syracuse, NY) or obtained from Dr. A. Fuji (Japan Kaiko Co., Ltd, Tokyo, Japan). Dr. A. Fuji also kindly supplied us with the BLM dA2 (Lot TN-A-029) used in these experiments. Both BLM A2 and dA2 were stored as stock solutions (10-3 M) in glass-distilled water at −20°C, and their purity was monitored by HPLC (9). Lidoamine HCl (Lot A-2126) was the gift of Astra Pharmaceutical Products, Inc. (Worcester, MA), and solutions were made fresh prior to use. Clinical grade cis-diaminodichloroplatinum, mitomycin C, and etoposide were used. Talsiomytin Socr was supplied by Bristol Laboratories (Syracuse, NY). High-specific activity Cu(II) [dimethylsulfonium 3H] BLM A2 (43Ci/mmol) was synthesized by New England Nuclear (Boston, MA) and stored in the copper form at 4°C in 30% ethanol: H2O. This radiolabeled material comigrated with unlabeled BLM A2 on high-pressure liquid chromatography. Its in vivo pharmacokinetics and metabolism have been described previously (10). Measurements of Cellular Radioactivity. Exponentially growing L1210 cells were resuspended (7 × 105 cells/ml) in Fischer's medium with 10% horse serum and 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (pH 7.4). Initial studies in our laboratory indicated that 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid did not alter the cytotoxicity of BLM A2 to L1210 cells. [3H]BLM A2 was added to BLM A2 to yield a final concentration of 1 μM [3H]BLM A2 (4.3 μCi/ml; specific activity, 4.3 μCi/μmol) which was added to cells in the presence or absence of 8 mM lidocaine. Cells were incubated with periodic shaking at 37°C in a water bath for 1 h. At the end of the incubation, 15 ml of an ice-cold phosphate-buffered 0.9% NaCl solution was added to each cell suspension, and, after centrifugation at 1000 × g for 2 ml, the supernatant fraction was removed, and the cell pellet was resuspended in 350 μl of Fischer's medium with 10% horse serum. The cell concentration of this suspension was determined with a Coulter counter, and triplicate samples of cells (100 μl each) were placed in a microfuge tube containing 40 μl of 5% HClO4 overlayed with 400 μl of silicone oil/mineral oil (84:16 v/v) mixture. The microfuge tubes were then centrifuged at 9000 × g for 15 s, and the tubes were rapidly frozen in an ethanol/dry ice bath, cut through the oil layer, and the radioactivity in the bottom layer of the tube (cell fraction) was counted. The rapid washing of cells with the phosphate-buffered 0.9% NaCl solution was necessary because of the small amount of radioactivity associated with cells and the relatively large amount of radioactivity associated with the extracellular water retained by the unwashed cells as they were centrifuged through the oil mixture. With L1210 cells incubated for 1 h with [3H]BLM A2, we have been unable to document any significant loss of cellular radioactivity during the rapid washing procedure. Furthermore, washing of cells does not appear to alter the cytotoxic actions of BLM A2 and, thus, the drug found associated with the cells is assumed to be pharmacologically important. Less than 1% of the total cellular radioactivity found in the cell pellet after washing with the NaCl solution and microcentrifugation was due to extracellular associated radioactivity. The radioactivity in the cell pellet was counted by liquid scintillation techniques and corrected to dpm by external standard techniques.

BLM Hydrolyase Assay. BLM A2 metabolism by cytosolic BLM hydrolyase was studied in vitro using our methods described previously (2, 9) and approximately 5 × 106 exponentially growing L1210 cells. The substrate concentration was 20 μg of BLM A2 per ml. Formation of BLM dA2 was determined by high-pressure liquid chromatography and fluorescence detection. Alkaline Elution Techniques. DNA strand breakage was determined by the methods of Kohn (8). L1210 cells were pretreated at 37°C with [3H]thymidine (0.01 μCi/ml, 1 μM) for 18 h. After the 18-h incubation, the medium was removed, and new medium supplemented with 1 μM unlabeled thymidine was added for 6 h. At the end of the 6 h, the medium was removed, and new medium with BLM A2 and/or lidocaine was added for 1 h. The medium was removed, and the cells were resuspended in ice-cold phosphate-buffered NaCl solution (pH 7.4). The cells were kept on ice in the dark until assayed by alkaline elution techniques. For the repair studies, cells were exposed to 25 μM BLM A2, washed with drug-free medium, resuspended in medium containing or lacking 8 mM lidocaine for 1 h, and then processed for the alkaline elution assay. In other studies, cells were pretreated with 8 mM lidocaine for 1 h, washed with drug-free medium, resuspended in medium containing 25 μM BLM A2 for 1 h, and then assayed for DNA breaks. Internal standard cells were prelabeled with [3H]thymidine (0.1 μCi/ml, 1 μM) in a similar manner and received a 300-rad dose at 4°C. Experimental and internal standard cells were admixed and deposited on a 2-μm filter (Nucleopore Corp., Pleasanton, CA). The cells were lysed with 5 ml of lysis buffer containing 2% sodium dodecyl sulfate, 20 mM EDTA, and 100 mM glycine (pH 10), and the eluate from the lysis procedure was collected and counted. The filters were then washed with 2 ml of the sodium dodecyl sulfate/EDTA glycine lysis buffer containing proteinase K (0.5 mg/ml; Sigma Co., St. Louis, MO). The elution solution (pH 12.1) of 40 ml of 0.1 M tetrapropyl ammonium hydroxide, 20 mM EDTA, and 0.1% sodium dodecyl sulfate was then added to each sample and pumped through the filter at approximately 2 ml/h directly into miniscintillation vials. Each fraction was collected for 90 min for a total period of 15 h. At the end of the collection, the excess eluting solution was removed. The filters were collected and hydrolyzed with 0.4 ml 1 N HCl for 1 h at 90°C, and the amount of radioactivity on the filter was determined. Finally, 2 ml of 1 M NaOH was pumped through the system and collected as a single fraction and counted. This fraction contained any residual radioactivity remaining
BLM AND LIDOCAINE

in the pump tubing or system.

The $^3$H and $^{14}C$ in each sample was determined simultaneously using dual labeling techniques. Correction for quenching was made using external standard methodology. Typical counting efficiencies for $^3$H and $^{14}C$ under the conditions described were approximately 30 and 70%, respectively. The total amount of $^{14}C$ and $^3$H in the sample was determined as the sum of the amounts in the individual fractions, the lysis fraction, the NaOH fraction, and the filter fraction. The fraction of $^{14}C$ retained (experimental cell DNA) was plotted against the fraction of $^3$H retained on the filter (internal standard DNA).

Plasmid DNA Breakage. The ability of lidocaine to influence DNA breakage by BLM A$_2$ was determined using a covalently closed circular DNA assay (11, 12). Form I plasmid pAT 153 DNA was isolated (13) and stored as a stock solution at 4°C in 40 mM Tris-acetate (pH 8.0) and 1 mM EDTA. Immediately prior to use, the stock DNA solution was diluted 4-fold with a 40 mM Tris-acetate solution (pH 8.0) to decrease the EDTA concentration. The incubation mixture (20 μl) contained 1 μg of DNA, 25 mM dithiothreitol, 20 mM MgCl$_2$, 40 mM Tris-acetate (pH 8.0), and BLM A$_2$ with or without 8 mM lidocaine. The molar ratio of BLM A$_2$ to pAT 153 DNA ranged from 0.3 to 100. The molecular mass of pAT 153 is 2.3 x 10$^6$ daltons. The incubation was conducted for 5 min at 4°C and stopped with the addition of 50 mM EDTA. The disappearance of form I DNA and the appearance of forms II and III DNA were assayed by gel electrophoresis with 1% agarose gels at room temperature for 4 h at 4.5 V/cm. Following electrophoresis, the gels were stained with ethidium bromide (1 μg/ml) in 40 mM Tris-acetate for 30 min (12), and the DNA was visualized by UV irradiation.

RESULTS

Potentiation of BLM A$_2$ Cytotoxicity by Lidocaine. Incubation of L1210 cells for 1 h with 0.1 to 10 mM lidocaine produced no decrease in clonogenicity (Chart 1). When cells were exposed to 5 μM BLM A$_2$ alone for 1 h, clonogenicity was decreased to 43 ± 6% of untreated control cells. Addition of lidocaine (0.1 to 10 mM) to cells incubated with 5 μM BLM A$_2$ caused a concentration-dependent potentiation of the BLM A$_2$ cytotoxicity.

The enhancement of BLM A$_2$ cytotoxicity by lidocaine also was dependent upon the concentration of BLM A$_2$. Incubation of L1210 cells for 1 h with 1 μM or less of BLM A$_2$ in the absence of lidocaine produced little cytotoxicity, while significant reductions in cell survival were seen with concentrations of BLM A$_2$ >5 μM (Chart 2). A significant increase in BLM A$_2$ cytotoxicity was observed with all concentrations of BLM A$_2$ (0.1 to 10 μM) in the presence of 8 mM lidocaine. Incubation of cells for 1 h with 10 μM BLM A$_2$ and 8 mM lidocaine caused a 1000-fold increase in cell kill compared to exposure to 10 μM BLM A$_2$ alone. Addition of lidocaine (8 mM) decreased the concentration of BLM A$_2$ required for 90% cell kill by approximately 40-fold. Due to the detection limits of our assay, we were unable to estimate accurately the cell survival after treatment with 8 mM lidocaine and 30 μM BLM A$_2$, but lidocaine clearly enhanced the cytotoxicity seen with 30 μM BLM A$_2$ by more than 8000-fold. The lidocaine potentiation of BLM A$_2$ cytotoxicity was not limited to the murine L1210 leukemic cells. The cytotoxicity of BLM A$_2$ toward a human head and neck carcinoma (A-253), which was more sensitive to BLM A$_2$ than were L1210 cells, was also enhanced (Table 1), although the potentiation was not as marked at this concentration as was that seen with L1210 cells.

Drug Sequence Effects. We next studied the effect of pre-
Table 1

<table>
<thead>
<tr>
<th>Condition</th>
<th>% of untreated control</th>
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<tr>
<td>Control</td>
<td>100 ± 7.0^a</td>
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<tr>
<td>Lidocaine (8 mM)</td>
<td>124 ± 26</td>
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<tr>
<td>BLM A2 (0.7 μM)</td>
<td>33.3 ± 2.4</td>
</tr>
<tr>
<td>BLM A2 (0.7 μM) + Lidocaine (8 mM)</td>
<td>14.3 ± 1.0^b</td>
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^a Mean ± SE; n = 6 to 9.

BLM and LIDOCAINE

Table 1

Potentiation of BLM A₂ cytotoxicity by lidocaine in human A-253 cells

Human head and neck tumor cells (A-253) were detached and exposed to BLM A₂ and/or lidocaine for 1 h. After washing with drug-free medium, 10^5 to 10^6 cells were placed in culture dishes containing soft agar and allowed to grow for 12 days at 37°C. Colony formation in untreated control was 142 ± 10 per 10^5 cells plated.

<table>
<thead>
<tr>
<th>Condition</th>
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<tbody>
<tr>
<td>Control</td>
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<tr>
<td>BLM A₂ (0.7 μM) + Lidocaine (8 mM)</td>
<td>14.3 ± 1.0^b</td>
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^a Mean ± SE; n = 6 to 9.

or postincubation of L1210 cells with lidocaine on BLM A₂ cytotoxicity. Chart 3, A and B, shows the protocol and results from experiments in which 5 μM BLM A₂ was added at various times before, during, or after a 1-h incubation with 8 mM lidocaine. Cells pretreated with lidocaine for 1 h followed immediately by BLM A₂ exposure (Chart 3B, value at e) showed only a limited difference in cell kill compared to BLM A₂ treatment alone (24.0 versus 46.8%) and a marked decrease in potentiation compared to that seen when the 2 agents were added simultaneously (Chart 3B, value at d) (24.0 versus 0.2%). An even smaller enhancement in BLM A₂ cytotoxicity was observed when cells were incubated for 0.5 or 1 h in drug-free medium after removal of lidocaine (Chart 3B, values at f and g). In contrast, preincubation of cells with BLM A₂ 1 h before lidocaine treatment (Chart 3B, value at c) caused an enhancement of BLM A₂ cytotoxicity that almost equaled that detected when the cells were coincubated with the 2 agents (Chart 3B, value at d) (24.0 versus 0.2%). An even smaller enhancement in BLM A₂ cytotoxicity was observed when cells were incubated for 0.5 or 1 h in drug-free medium after removal of lidocaine (Chart 3B, values at f and g). In contrast, preincubation of cells with BLM A₂ 1 h before lidocaine treatment (Chart 3B, value at c) caused an enhancement of BLM A₂ cytotoxicity that almost equaled that detected when the cells were coincubated with the 2 agents (Chart 3B, value at d). The cells appeared, however, to have a poor memory for prior BLM A₂ exposure; a decrease in the potentiation was noted if a 0.5- or 1-h drug-free interval was interspaced between the BLM A₂ and lidocaine treatment (Chart 3B, values at a and b). Thus, these data indicate that marked potentiation occurs only when lidocaine is present with or immediately after BLM A₂ exposure and suggest that lidocaine does not augment the cytotoxicity of BLM A₂ by enhancing BLM A₂ uptake.

Drug Specificity of Lidocaine Potentiation. To determine if the potentiation seen with lidocaine was specific for BLM A₂, we examined other antitumor agents known to interact with DNA. Incubation of L1210 cells with 8 mM lidocaine for 1 h with 2 μM mitomycin C caused no statistically significant increase in toxicity (Table 2). Coincubation with 8 mM lidocaine and 1 μM etoposide did result in a slight potentiation of etoposide cytotoxicity, but we do not believe this has any biological significance, because the magnitude of the change is so small. With cis-diamminedichloroplatinum, a larger increase in cytotoxicity was noted, although this was small compared to that seen with BLM A₂ or talismycin S10b. These data suggest that the lidocaine potentiation phenomenon is restricted to a subset of DNA-interacting agents and that BLM analogues with modifications in the C-terminus and near the bitiazole region of the molecule retain the property of enhancement by lidocaine.

DNA Breakage. We next evaluated the effect of lidocaine on BLM A₂-induced DNA strand breaks in intact cells. Exposure of L1210 cells to either 5 μM BLM A₂ alone (data not shown) or 8 mM lidocaine alone (Chart 4) for 1 h produced no evidence of significant DNA breakage compared to untreated control cells. With 10 μM BLM A₂ alone, the average amount of single strand breaks was calculated to be 5 rad-equivalents, whereas cells treated concurrently with the combination of 10 μM BLM A₂ and 8 mM lidocaine had an average DNA strand breakage of 56 rad-equivalents. This represents at least a 10-fold increase in the amount of DNA strand breakage.

The effect of lidocaine exposure on the DNA integrity of BLM A₂ pretreated L1210 cells was also examined. Incubation of cells treated for 1 h with 25 μM BLM A₂ resulted in an average DNA strand breakage of approximately 80 rad-equivalents. These values represent at least a 10-fold increase in the amount of DNA strand breakage.
Table 2
Lidocaine interactions with other antitumor agents

L1210 cells were incubated for 1 h with 1 µM of each antitumor agent, except mitomycin C, which was 2 µM in the absence or presence of 8 mM lidocaine. Cells were washed free of drugs, and survival was determined by clonal growth in soft agar. Each value is the mean from 3 to 6 determinations.

<table>
<thead>
<tr>
<th>Survival (% of untreated control) with lidocaine (mM)</th>
<th>0</th>
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<tbody>
<tr>
<td>Untreated</td>
<td>100 ± 2</td>
<td>97 ± 2</td>
</tr>
<tr>
<td>Mitomycin C</td>
<td>93 ± 2</td>
<td>89 ± 6</td>
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<tr>
<td>cis-Diamminedicloroplatinum</td>
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<tr>
<td>Talismycin Si2</td>
<td>88 ± 5</td>
<td>3 ± 1</td>
</tr>
<tr>
<td>Etoposide</td>
<td>18 ± 1</td>
<td>13 ± 0.4</td>
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</table>

* Mean ± SE.

Although in no experiments did the DNA integrity return to control values. Inclusion of 8 mM lidocaine during this repair period not only blocked the repair of DNA but caused a 4.4-fold increase in DNA strand breakage (350 rad-equivalents) consistent with the enhanced cell death that was observed with this drug exposure sequence (Chart 3). Using a treatment schedule similar to that which did not cause a marked increase in BLM cytotoxicity (Chart 3, value at e, i.e., 1 h exposure to 8 mM lidocaine followed by a 1-h exposure to 25 µM BLM A2), DNA strand breakage was not significantly increased compared to that seen when cells were exposed to BLM A2 alone, i.e., 90 versus 80 rad-equivalents, respectively (data not shown).

To determine if lidocaine enhanced the ability of BLM A2 to cause single- and double-strand breaks to isolated DNA, we next exposed covalently closed circular pAT 153 DNA (form I) to various concentrations of BLM A2. After incubation, the untreated DNA displayed primarily the closed circular form I DNA. Addition of 8 mM lidocaine alone (data not shown) indicated that lidocaine did not cause DNA breaks. Formation of form II DNA became noticeable with molar ratios of BLM A2 to DNA of 4:1. Higher ratios of BLM A2 to DNA (e.g., 100:1) resulted in the appearance of form III DNA and the more complete degradation of pAT 153 DNA. The inclusion of 8 mM lidocaine did not increase BLM A2-induced DNA breakage. Thus, the enhanced breakage seen in L1210 cell DNA appears not to be mediated by a direct effect of lidocaine on BLM A2-induced DNA damage.

Cellular Content of BLM A2. The effect of lidocaine exposure on BLM A2 association with L1210 cells was examined using [3H]BLM A2. We incubated L1210 cells with 1 µM [3H]BLM A2 for the same 1-h period used previously in the cytotoxicity studies. Cells were rapidly washed with an ice-cold phosphate-buffered 0.9% NaCl solution and then centrifuged through an oil mixture. Results with HeLa cells (19) and L1210 cells indicated that cellular accumulation of radioactivity occurred rapidly and, at 1 h, an approximate steady state existed. When cells were incubated for 1 h with 8 mM lidocaine and 1 µM [3H]BLM A2, there was 57% less radioactivity associated with the cells compared to that observed with cells incubated without lidocaine [1160 ± 90 dpm/10⁶ (treated cells) versus 2720 ± 80 dpm/10⁶ (control cells)]. Thus, it is unlikely that lidocaine augments the cytotoxic actions of BLM A2 by increasing the total cellular content of drug.

BLM A2 Metabolism. The possibility of an interaction between lidocaine and BLM metabolism was studied with an in vitro assay of BLM hydrolase. Using homogenates from L1210 cells and a substrate concentration of 20 µg/ml, the formation of BLM dA2 was linear for at least 2 h, occurring at a rate of 1.85 ± 0.08 µg BLM dA2 formed/h/mg protein (Chart 6). Addition of 8 mM lidocaine to the incubation caused a small but significant decrease in the initial rate of BLM dA2 formation. The effect of various concentrations of lidocaine on in vitro BLM A2 metabolism was determined next (Table 3). The maximum inhibition observed was 24%, and it occurred with 16 mM lidocaine. The use of higher BLM A2 substrate concentrations (≥50 µg/ml) resulted in
Lidocaine. Control activity was the rate of BLM dA2 formation in homogenates incubated with BLM A2 (20 μg/ml) for 30 to 90 min with various concentrations of crude homogenates. Nonetheless, these results did indicate that the enzyme activity in the homogenate did not comply with Michaelis-Menten kinetics at substrate concentrations below 50 μg/ml, possibly due to the presence of binding proteins in the presence (A) of 8 mw lidocaine. Results are taken from 3 experiments. Each value is the mean of 3 or more determinations; bars, SE.

Table 3

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<tr>
<th>Lidocaine (mm)</th>
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<td>16</td>
<td>75.7 ± 2.7 (8)</td>
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</table>

* Mean ± SE.
* Numbers in parentheses, number of determinations.
* P < 0.05 compared to control.

Chart 6. Effect of 8 mw lidocaine on the initial rate of BLM dA2 formation in vitro. The 105,000 x g supernatant fraction from exponentially growing L1210 cells was assayed by HPLC for bleomycin hydrolase activity by incubating protein (1.6 mg/ml) with BLM A2 (20 μg/ml) for various times at 37°C in the absence (○) or presence (O) of 8 mw lidocaine. Results are taken from 3 experiments. Each value is the mean of 3 or more determinations; bars, SE.

Inhibition of the rate of BLM dA2 formation by lidocaine in vitro

Initial rate of BLM dA2 formation in 105,000 x g supernatant fractions of cultured L1210 cells was assayed by HPLC. Homogenates (1.6 mg protein/ml) were incubated with BLM A2 (20 μg/ml) for 30 to 90 min with various concentrations of lidocaine. Control activity was the rate of BLM dA2 formation in homogenates lacking lidocaine.

Chart 7. Effect of lidocaine on the cytotoxicity of BLM dA2. Exponentially growing L1210 cells were incubated for 1 h with various concentrations of BLM dA2 in the absence (○) or presence (O) of 8 mw lidocaine. Cells were washed free of drugs, and survival was measured by colony formation in soft agar. Each value is the mean from a minimum of 3 culture tubes; bars, SE.

Lidocaine Interactions with BLM dA2. Previous results from Umezawa et al. (23) indicated that the product of BLM hydrolase, the desamido metabolite, was less active as an antimicrobial agent and caused fewer single- and double-strand DNA breaks. Consistent with these previous observations, only high concentrations of BLM dA2 were cytotoxic to L1210 cells (Chart 7). A 1 h incubation with 50 μM BLM dA2 caused only a 20% reduction in L1210 cell survival. Addition of 8 mm lidocaine, however, enhanced cell kill with 1, 10, and 20 μM BLM dA2, bringing the values close to that seen with parent drug in the absence of lidocaine. These results demonstrate that even BLM analogues with modifications on the N-terminus can be potentiated by lidocaine. Furthermore, they indicate that inhibition of BLM metabolism may not be the only mechanism by which lidocaine could act.

**DISCUSSION**

The ability of a number of agents to enhance the cytotoxicity of clinical grade BLM or peplomycin has been reported by several investigators (3, 6, 16–18). These agents include polyene antibiotics (1), hyperthermia, and ethanol exposure (6, 14, 17). The mechanism responsible for potentiation by the polyene antibiotics appears to be due to elevated cellular BLM content, while the mechanisms for enhancement with ethanol and hyperthermia are not known. Our results, which confirm those of Mizuno et al. (15), indicate that local anesthetics such as lidocaine can greatly enhance the cytotoxicity of the BLM class of compounds. The enhancement occurs with analogues having modifications on either the C- or N-terminal moiety. Moreover, at least limited additions to the domain close to the bithiazole moiety, such as those present in talisomycin S10b, can be tolerated. Unlike the polyene antibiotics, lidocaine does not appear to elevate the cellular content of BLM A2. The potentiation seen with lidocaine is also different from that seen with ethanol, since enhancement was greater when cells were treated with ethanol prior to or after BLM treatment compared to coincubation (14). The reported potentiation with hyperthermia also differs from our results with lidocaine, because preheating was significantly more effective than postheating (14).

The enhanced cytotoxicity as measured by soft agar assay after coincubation with BLM A2 and lidocaine reflected an enhanced DNA damage as detectable by alkaline elution. Incubation of L1210 cells with 10 μM and 25 μM of BLM A2 alone for 1 h resulted in approximately 5 and 80 rad-equivalents of DNA damage, respectively. While seemingly small, it has been observed previously that high concentrations of BLM are required to detect DNA strand breakage in intact cultured cells (7). This may reflect a rapid repair system. It is also possible that noncovalent DNA cross-linking (11) may occur which retards the elution of DNA fragments causing anomalously slower elution times. It is evident, however, that coincubation of L1210 cells with lidocaine results in more rapid elution of DNA, suggesting greater DNA strand breaks. Furthermore, addition of lidocaine to BLM A2-pretreated cells greatly potentiated the DNA damage (4.4-fold), while addition of BLM A2 to lidocaine-pretreated cells caused no increase in DNA breakage. Our results with pAT 153 DNA indicate that the greater DNA breaks are not the product of a more effective formation of radicals by BLM A2.
The enhancement seen with lidocaine is relatively specific for the bleomycin class of compounds, although a small enhancement with cis-diaminedichloroplatinum was noted. This selectivity for the bleomycin class may reside partially in the ability of lidocaine to inhibit BLM inactivation and in allowing a greater amount of biologically active drug to remain in cells. The enhanced DNA breakage and cytotoxicity seen with lidocaine exposure to BLM A₂-pretreated cells (Charts 3 and 5) may reflect inhibition in the metabolism of BLM A₂ that fails to leave cells after washing (19). Quantitatively, however, the in vitro inhibition of bleomycin hydrolysis with 8 mM lidocaine was small (<25% inhibition), and lidocaine also enhanced the cytotoxicity of cis-diaminedichloroplatinum and the product of BLM hydrolysis, BLM dA₂. Thus, it appears that another mechanism must also be operative.

Lidocaine has been shown to alter the excision repair process in procaryotic cells (22). The repair of DNA damage after bleomycin treatment is known to occur quite rapidly (7). In L1210 cells pretreated with 25 μM BLM A₂, the DNA repair seen after a 1-h incubation in drug-free medium is abolished with lidocaine. Thus, lidocaine may also act to inhibit the repair of DNA damage in L1210 cells treated with BLM A₂. Calmodulin inhibitors have been reported to inhibit the repair of BLM-induced DNA damage (3), and lidocaine has been reported to be a calmodulin inhibitor (21). Using L1210 cell calmodulin and phosphodiesterase, however, we have not been successful in demonstrating inhibition of calmodulin-dependent enzyme activity with lidocaine. Thus, it seems unlikely that lidocaine acts via inhibition of a calmodulin-mediated event.

Our results with both murine and human tumor cells demonstrate that the DNA damage and cytotoxicity of the BLM class of antitumor glycopeptides can be profoundly influenced by the presence of the local anesthetic lidocaine. The mechanism for this enhancement may reside partially in the ability of the local anesthetic to block the metabolic inactivation of BLM and the repair of BLM-induced DNA damage.

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7 J. S. Lazo and W. N. Hait, unpublished observation.
Lidocaine Potentiation of Bleomycin A2 Cytotoxicity and DNA Strand Breakage in L1210 and Human A-253 Cells

John S. Lazo, I. Deborah Braun, Borislav Meandzija, et al.


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