Liblomycin-mediated DNA Cleavage in Human Head and Neck Squamous Carcinoma Cells and Purified DNA


ABSTRACT

Liblomycin (LBM), a novel bleomycin analogue, and bleomycin A2 (BLM A2) were compared with respect to their relative potential to inhibit growth in a human head and neck squamous carcinoma cell line and to produce DNA damage within cellular DNA and nuclei DNA and against isolated naked DNA. Against the BLM-sensitive cell line 183A, the concentration of LBM that inhibits cell growth by 50% was 1.1 μM for a 30-min drug exposure, while it was 23 μM for BLM A2. Drug-mediated DNA double-strand cleavage within cells was compared with the relative ability of these drugs to produce DNA cleavage in isolated 183A cell nuclei. Though 30-min exposures of cells to equimolar concentrations of both drugs resulted in 4-fold greater cellular DNA damage by LBM than BLM A2, the two drugs were nearly equipotent in producing DNA injury within isolated nuclei. Against Simian virus 40 DNA, however, LBM was 10-fold less effective than BLM A2 in producing Forms II and III DNA from Form I DNA. Radioactivity from either [3H]BLM A2 or 125I-LBM found associated with cells after a 30-min incubation period was also assessed in the 183A cell line. The exposure of cells to radiolabeled drug (1 μM) resulted in a 71-fold greater amount of cell-associated radioactivity for LBM than for BLM A2. The relative abilities of the 183A cell line to partially reseal LBM- or BLM A2-mediated DNA double-strand breaks were also assessed. No preferential repair of overall drug-mediated DNA injury, however, was observed. Finally, drug-mediated specific cleavage sites on pBR322 DNA were determined. At doses that gave the same extent of DNA cleavage, both BLM A2 and LBM gave similar patterns of strand scission, although minor differences were observed. Taken together, these data demonstrate that the greater efficacy of LBM against the BLM-sensitive head and neck squamous cell line is due mainly to LBM's greater association with cells over a defined time period, even though the DNA cleaving ability of LBM is relatively lower than that of BLM A2.

INTRODUCTION

In 1985 Umezawa et al. (1) announced the synthesis and initial preclinical results of a new bleomycin analogue, LBM, which possessed greater antitumor activity than did BLM and which was also reported to lack any significant potential to produce pulmonary injury. Our recent studies have also shown that LBM is a unique BLM analogue. For example, it retains activity against BLM-resistant tumors (2) and may possess a different spectrum of antitumor efficacy based upon a recent report of its cytotoxicity relative to BLM against a number of different human tumors in a clonogenic assay (3). We have also reported that LBM shows little evidence of histopathological injury in rodent lungs, in sharp contrast to the severe injury produced by therapeutic doses of BLM (4). A recent report of the clinical Phase I trial of LBM (5) also confirmed the very limited potential of this compound to produce pulmonary injury which, in turn, suggests that clinical trials in the United States are warranted.

In an attempt to understand the apparent differences between tumor response to LBM and to BLM, we investigated the biochemical pharmacology of this novel BLM analogue. Specifically, we determined the abilities of LBM and BLM A2 (the principal component of the natural bleomycin mixture) to produce DNA cleavage in a human head and neck carcinoma cell line, isolated nuclei, and SV40 DNA. In addition, we determined cleavage specificity patterns of LBM and BLM A2 using a defined segment of pBR322 DNA.

MATERIALS AND METHODS

Chemicals. [2-14C]Thymidine (≥50 μCi/mmol) and [S-methyl-3H]-BLM A2 (44.1 Ci/mmol) were purchased from New England Nuclear, Boston, MA. Simian virus 40 DNA (Form I) was purchased from Lofstrand Labs. Limited, Gaithersburg, MD. HindIII, EcoRI, pBR322, and λ-DNA were obtained from Pharmacia, Inc., Piscataway, NJ. LBM was a kind gift from Dr. T. Takita (Nippon Kayaku Co., Ltd., Tokyo, Japan), and BLM A2 was a gift from Dr. T. T. Sakai (Comprehensive Cancer Center, University of Alabama, Birmingham, AL). Immediately prior to use, drugs were dissolved in 0.9% sodium chloride.

LBM was labeled with 125I using chloramine T (6) following a modified method of Broughton and Strong (7). To 10 μl of 1 mCi of Na125I, 10 μl of chloramine T (5 mg/ml) and 10 μl of BLM (1 mg/ml) were added, gently mixed, and incubated for 10 min at room temperature. The reaction was stopped by adding 10 μl each of sodium metabisulfite (12 mg/ml) and potassium iodide (20 mg/ml). All the solutions were made in 0.1 M borate buffer (pH 9.0). The mixture was then applied on a cation exchange column of CM-Sephadex C-25 (20 x 0.9 cm). The unreacted Na125I was eluted with 0.1 m ammonium formate (pH 6.4). The 125I-labeled and unreacted LBM was then eluted with 1 m ammonium formate (pH 6.4). Radioactive LBM was either used immediately or frozen at −70°C and subsequently used within 3 days after preparation. Recovery of labeled LBM was essentially complete from the column, and the resulting 125I-LBM had a specific activity of 560 mCi/mmol.

Cell Lines. The human head and neck squamous cell carcinoma cell line, 183A, was the kind gift of Dr. Peter G. Sacks (Department of Tumor Biology, M. D. Anderson Cancer Center). The in vitro characteristics and cytogenetic patterns of this cell line have been described in detail elsewhere (8). Cell cultures were maintained at 37°C in a humidified atmosphere of 95% air:5% CO2 in Dulbecco's modified Eagle's medium (Gibco Laboratories, Grand Island, NY) supplemented with 10% fetal bovine serum (Hazleton Research Products, Inc., Denver, PA), penicillin (100 units/ml), streptomycin (100 μg/ml), amphotericin B (0.5 μg/ml), and l-glutamine (584 μg/ml). Cell cultures of
183A for all experiments in this study were in the exponential phase of growth.

Assessment of Cell Growth. Single cell suspensions of 5 x 10^4 cells/ml were obtained by gentle trypsinization of adherent cultures. Cells were plated in Falcon 96-well microtiter plates (Becton Dickinson Labware, Lincoln Park, NJ) in a volume of 80 µl/well. After a 72-h incubation at 37°C, LBM or BLM was added at different concentrations, and the cells were further incubated at 37°C for 30 min. Following drug exposure, cells were washed twice with ice-cold HBSS (Gibco Laboratories, Grand Island, NY) and reincubated in drug-free medium (80 µl/well). At 55 h after drug treatment, a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide cell growth assay was performed using a modification (9) of the procedure originally described by Mosmann (10). All results represent the average of a minimum of 8 replicate wells per experiment. Individual experiments were repeated at least 3 times.

Isolation of Cell Nuclei. Single cell suspensions of 183A cells were obtained by gentle trypsinization, then centrifuged, and resuspended in nuclei buffer containing 0.3% Triton X-100, then centrifuged, and resuspended in 4°C as described previously (11). Cells were then gently diluted in 10 volumes of nuclei buffer without detergent. Nuclei were obtained by gentle trypsinization, then centrifuged, and resuspended in 0.1% SDS. Horizontal gel electrophoresis was carried out at room temperature (25 V, 16 h) in a buffer containing 9 mM Tris, 89 mM boric acid, and 1 mM disodium EDTA (pH 8.0). After electrophoresis, the gels were stained in the electrophoresis buffer containing ethidium bromide (1 µg/ml) for a minimum of 30 min. Gels were then visualized (UV light box) and photographed with Polaroid 55 or 57 films. The order of migration of DNA (fastest to slowest) under these conditions was covalently closed circular DNA (Form I), double-strand broken linear DNA (Form III), and single-strand broken circular DNA (Form II). Quantitative measurement of the amounts of Forms I, II, and III SV40 DNA that had migrated into the gel was derived from densitometer scanning (Beckman DU 8B) of the negative of a given gel photograph.

Rescaling of DSB. The 183A cells were treated with either LBM or BLM A2 for 30 min at a concentration that induced the same initial amount of DSBs, i.e., 5 µM LBM and 15 µM BLM A2. Cells were then washed free of drug twice with 37°C medium. Incubation was continued in drug-free medium at 37°C for the indicated period of time, and the cells were then removed from the flasks with a trypsin-EDTA solution on ice. Hereafter, the cells were processed as described for the filter elution assay.

Cell-associated Radioactivity Studies. One h prior to beginning cell-associated radioactivity experiments, the medium for exponentially growing 183A cells was changed to fresh medium containing 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid. Cells were then exposed to LBM or BLM for 5 or 30 min at 37°C. Drug uptake was stopped by the three rapid washes of cold HBSS, after which the cells were detached by incubation with trypsin and EDTA at 37°C. Radioactivity was measured by either a liquid scintillation spectrometer for [3H]LBM A2-treated cells after solubilization with a sonicator or by a γ scintillation counter for [3H]LBM A2-treated cells.

DNA Cleavage Specificity. HindIII-digested pBR322 DNA was labeled at its 5’ ends (15) and further digested with EcoRI. Digestion of the radiolabeled DNA (approximately 10,000 cpm/sample) by BLM A2 or BLM was carried out in a solution of 20 mM NaCl and 10 mM Tris (pH 7.4) containing 50 µM base pair λ-DNA, 10 µM Fe(III), and 3 mM diethiothreitol in a volume of 20 µl. Samples were incubated for 30 min at room temperature prior to freezing and lyophilization.

The dried samples were dissolved in 2 µl of sample solvent (80% formamide:1 mM EDTA:0.1% bromophenol blue:0.1% xylene cyanol) and heated at 90°C for 1 min prior to chilling on ice. Samples were loaded onto a 6% polyacrylamide sequencing gel and run at 2500 V at 55°C for approximately 1.5 h on an LKB Macrophor electrophoresis unit. The gel was transferred onto filter paper and placed in contact with Kodak XAR X-ray film and stored at ~70°C for 24 h. After development, the film was scanned using an LKB Ultrascan XL laser densitometer.

RESULTS

Dose-dependent Survival. ICS0 data derived from studies of the inhibition of growth of 183A cells as a function of LBM and BLM A2 concentrations are presented in Table 1. These values were estimated to be 23 µM for BLM A2 and 1.1 µM for BLM, demonstrating that BLM is about 20-fold more potent than BLM in this cell line.

Drug-mediated DNA Damage. To ascertain whether drug-mediated growth-inhibitory effects were accompanied by production of DNA DSBs within cells, the DNA double-strand cleaving activity of the drugs was assessed using a neutral filter elution assay. Experiments were performed after treatment of whole cells with either LBM or BLM A2 for 30 min at 37°C. Both drugs produced DNA DSBs in 183A cells (Fig. 1). The...
cleavage frequency per µmol of drug per 30-min treatment period was 1234 rad equivalents for LBM and 313 rad equivalents for BLM A2, respectively (see Table 1). Single-strand DNA breaks were also measured in intact cells. Three-fold more SSBs were produced by LBM than BLM A2 in intact cells.

DNA DSBs in drug-treated isolated nuclei of 183A cells were then quantified. DNA DSBs produced by LBM and BLM A2 were linearly related to the applied drug treatment (Fig. 2). The estimated values for production of DNA DSBs were 411 rad equivalents/µmol of LBM and 329 rad equivalents/µmol of BLM A2, demonstrating that the potencies of the two drugs in nuclei of 183A cells were similar (Table 1). This observation differs from that of the relative effects of these two drugs in intact 183A cells where equimolar concentrations of LBM produced a 4-fold greater extent of DNA cleavage than did BLM.

DNA Cleavage. The relative abilities of LBM and BLM A2 to convert supercoiled double-stranded DNA to nicked circular DNA and linear-duplex DNA were measured as an indication of the in vitro DNA cleavage potential of these drugs. Control experiments (without drug) resulted in no DNA degradation per se, although a small fraction of SV40 Form I DNA degraded during isolation and handling was observed in a few control experiments (Fig. 3, Lane A). Control incubations with 50 µM Fe(III) and 25 mM β-mercaptoethanol resulted in no degradation of SV40 DNA (Fig. 3, Lane C). When reaction mixtures containing Fe(III), β-mercaptoethanol, and SV40 Form I DNA were treated with either LBM (Fig. 3, Lanes D to G) or BLM A2 (Lanes I to L), a progressive conversion to Form II and Form III was observed. The extent of conversion was proportional to the amount of drug added. In experiments carried out without exogenously added metal, the presence of either LBM or BLM A2 did not result in any further strand scission compared with that of controls (Fig. 3, Lanes H and M). In each case, the extent of strand scission was clearly enhanced by subsequent addition of Fe(III).

Fig. 4 shows the fraction of Form II plus Form III DNA produced by increasing concentrations of either LBM or BLM A2. Those drug concentrations that resulted in a 50% reduction of Form I DNA were estimated to be 0.3 µM and 3.8 µM for BLM A2 and LBM, respectively. The extent of production of Form III DNA relative to Form II DNA at 50% reduction in Form I DNA was of the same order of magnitude for the two compounds (0.15 and 0.23 for BLM A2 and LBM, respectively).

Cell-associated Radioactivity. The association of radiolabeled BLM A2 with LBM was compared in 183A cells after exposure to 1 µM [3H]BLM A2 or [125I]LBM for 30 min at 37°C. As indicated in Table 1, the relative amount of labeled LBM was markedly higher than that of BLM A2. The ratio of incorporated amounts of LBM to BLM A2 after 30-min exposure was 71, indicating a much greater extent of cell-associated LBM than BLM.

Reversal of Drug-mediated DNA Cleavage. The relative ability
Fig. 4. DNA cleavage activities of LBM (O) and BLM A1 (•). The reaction mixtures containing various concentrations of drug, 50 μM Fe(III), 25 mM β-mercaptoethanol, and 0.3 μg of SV40 in nucleus buffer (pH 6.4) were incubated at 37°C for 30 min as described in "Materials and Methods." After agarose gel electrophoresis, gels were stained with ethidium bromide and photographed. The film negative of the gel photograph was used for quantitation of Forms I, II, and III DNA by densitometry. Individual points are shown for one to three experiments.

Fig. 5. Formation of DNA DSBs and repair kinetics in 183A cells. Cells with [14C]thymidine-labeled DNA were treated at 37°C for 30 min with 5 μM LBM (C) or 15 μM BLM A1 (•). Cells were then washed free of drug prior to neutral filter elution. Data represent the mean of two to three independent experiments.

of 183A cells to partially reseal LBM- or BLM-mediated DNA DSBs is shown in Fig. 5. Cells exposed to LBM or BLM A1 were washed and incubated further at 37°C in the absence of drug after treatments which yielded comparable initial frequencies of DNA DSBs for both agents. Following the end of the incubation period, cells were harvested with ice-cold trypsin-EDTA. The extent of DNA DSBs remaining after 3 h of drug-free incubation was about 25% in 183A cells treated initially with either LBM or BLM A1. The kinetics of DSB resealing were biphasic with an initial rapid component followed by a slower rate of DNA DSB removal.

DNA Cleavage Specificity of LBM and BLM. To compare the specific cleavage sites on DNA produced by BLM A1 and LBM, isotopically labeled DNA fragments were prepared as described in "Materials and Methods." The DNA cleavage specificity of BLM A1 and LBM on a defined sequence of pBR322 DNA is seen in Fig. 6. The peaks observed on the densitometric traces from the sequencing gel autoradiogram show the positions of cleavage, and the intensity of the peaks indicates the relative extent of cleavage at a particular site (Fig. 6A). The pattern of cleavage is shown schematically in Fig. 6B. Both drugs cleave DNA in the presence of Fe(III) and dithiothreitol, but a 20-fold higher concentration of LBM was required to produce the same overall extent of cleavage. Cleavage was observed at all 5'-GPy-3' sites along the sequence, but the intensity of cleavage between different sites varied up to 30-fold. The two occurrences of TGT and two of GGT in the sequence were cleaved preferentially. At doses that gave the same extent of DNA cleavage, both BLM A1 and LBM gave similar patterns of strand scission, although minor differences were observed (e.g., at positions 84 and 155).

DISCUSSION

Although some controversy exists (16, 17), it is generally assumed that the ability of BLM analogues to inhibit growth and produce cell death is associated with their ability to damage chromosomal DNA through either strand breaks (18-20) and/or through the release of nucleotide bases with the concomitant generation of alkaline-labile sites (21-25). In the present study, the relative abilities of LBM and BLM A1 to produce inhibition...
of cell growth were compared with their potential to cleave DNA within intact cells (Fig. 1). LBM has previously been reported to be more potent than BLM against a number of human and murine cell lines. Against the human head and neck squamous carcinoma cell line 183A, LBM was found to be 20-fold more potent on a molar basis than was BLM A2. Treatment of these cells with LBM resulted in a 4-fold greater number of DNA DSBs and a 3-fold increase in SSBs than was produced by an equiscytotoxic concentration of BLM A2.

The relative abilities of these drugs to produce cleavage of DNA within isolated nuclei (Fig. 2), SV40 DNA (Figs. 3 and 4), and pBR322 (Fig. 6), however, demonstrated that both drugs were nearly equivalent on an equivoluminar basis. In fact, against SV40 DNA, LBM proved to be less potent than was BLM A2. These findings raised an important question concerning the relative activity of drug within intact cells as opposed to cell-free systems. The greater cytotoxicity of LBM relative to BLM A2 against 183A cells could have several explanations including (a) greater drug entry and retention in cells, (b) differential cell repair of specific drug-mediated DNA injury, (c) altered cofactor requirements for each drug, (d) different cellular drug metabolism (ability of each drug to be metabolized by bleomycin hydrolase) or intracellular compartmentalization, and/or (e) qualitative differences in the formation of drug-mediated DNA lesions.

Because BLM and its analogues are believed to have an intracellular site of action, their relative abilities to cross biological membranes must be critical to their overall effectiveness as antitumor agents (17). Though the mechanism of cellular uptake of BLM is not clear, it is apparent that only a small amount of external drug actually enters intact cells (26). It is generally assumed that BLM crosses the membrane by slow passive diffusion (27). A relatively lipophilic BLM analogue such as LBM would therefore be expected to enter cells with greater ease than would BLM itself. In the present study, we observed that, within 30 min, the amount of cell-associated radioactive LBM exceeded that of BLM A2 by 71-fold. This finding agrees well with results published by Takahashi et al. (28). This group compared the cellular uptake of LBM with peplomycin and found that, on a equivoluminar basis, the amount of LBM associated with either BLM-sensitive or -resistant rat ascites hepatoma cell lines was over 100 times that of peplomycin. Thus, it appears that uptake per se to a large extent explains the greater cytotoxicity of LBM.

Other potential explanations for the differential cellular response to equimolar concentrations of LBM and BLM A2, however, were also investigated. Cell repair of drug-mediated DNA injury was determined using alkaline elution methodology. The data demonstrate that preferential repair of overall drug-mediated DNA lesions did not occur and thus cannot account for the greater cytotoxicity of LBM. In related studies, preliminary work in our laboratory was done to evaluate the relative abilities of LBM and BLM A2 to stimulate poly(adenosine diphosphoribose) synthesis, a process often associated with DNA repair. Both drugs were found to produce a significant but similar extent of stimulation of poly(adenosine diphosphoribose) synthesis in 183A cells. Thus, along with the DNA repair studies, the relative ability of cells to respond to individual drug-mediated injury by poly(adenosine diphosphoribose) synthesis does not appear to account for the relative differences in cytotoxicities between the drugs.

Recent studies by Smith (29) have suggested that differential cell responses to BLM can in part be attributed to either the relative endogenous level of Fe(II) or the cellular oxidation-reduction potential. Such observations may explain the relative differences in response of cell lines to BLM analogues; when drugs such as LBM and BLM A2 are compared within a single cell line, differences in cofactor requirements are unlikely to fully explain large differences in drug-mediated cytotoxicities. Comparisons of the relative abilities of BLM A2 and LBM to produce cleavage of SV40 DNA under defined conditions of iron concentration (Figs. 3 and 4; Refs. 1 and 30), in fact, suggest that LBM utilized Fe(II) 10-fold less efficiently than BLM A2, since the DNA cleaving activity of LBM is an order of magnitude lower than that of BLM A2 (Fig. 4). A thorough evaluation of the exact cofactor requirements of LBM, however, remains to be done.

Recent studies by Motegi et al. (31) have indicated that LBM is not a substrate for bleomycin hydrolase. Thus, one potential explanation for the greater cytotoxicity of LBM than BLM A2 in 183A cells could be the relative inactivation of BLM A2 by this cellular enzyme. While bleomycin hydrolase activity was not measured in the 183A cell line, similar human squamous head and neck cell lines have been shown to contain relatively low levels of bleomycin hydrolase activity (2). Thus, the relative presence and/or activity of bleomycin hydrolase within this specific cell line was not considered to be a primary factor in explaining the decreased response to BLM A2 relative to LBM.

Finally, the relative ability of BLM A2 and LBM to cleave pBR322 DNA was measured. Analysis of nucleotide sequence preference revealed that both LBM and BLM cleaved DNA most frequently at 5'-GC-3' and 5'-GT-3' sites, as has previously been observed for BLM (32, 33). The similar patterns of cleavage for LBM and BLM suggest that changes in the terminal amine moiety of BLM do not result in major alterations of site specificity of DNA fragmentation. This has also been observed for other BLM analogues (34). Although the present DNA cleavage sequence specificity studies were done with a pBR322 DNA sequence, these data are likely to reflect cleavage that occurs within the cell, since several investigators have now reported that BLM preferentially acts at the same positions in chromatin as it does in purified DNA (35, 36).

It appears that, for LBM and BLM A2, the extent of DNA cleavage is a more important determinant than is the actual DNA cleavage sequence specificity. In fact, Grimwalde et al. (36) have recently pointed out that a major difference in the cutting of purified and intracellular DNA by BLM appears to be the drug concentration required to achieve equivalent cleavage (37). Apparently, factors such as nucleoproteins and cell permeability can affect drug activity by reducing the quantity of drug-induced breaks but not by altering the site specificity of BLM-mediated cleavage (36). These studies are consistent with our present studies wherein the quantity of drug within the cell (and presumably the nucleus) appears to be of critical importance.

An interesting observation noted in the present study was a 3-fold higher frequency of DSBs in whole cells than in isolated nuclei following exposure to LBM, whereas the frequency of BLM-induced DSBs remained the same in these two systems. The calibration of the filter elution assay for DSBs was performed by subjecting whole cells or nuclei to a series of X-ray doses. These calibration lines were similar in whole cells and in isolated nuclei (as stated in "Materials and Methods") and indicate an independence of the extranuclear environment in X-ray-induced DNA strand breakage. Although the cellular metabolism of LBM is not fully understood, the observed difference in LBM-mediated DSBs in the two systems may be caused by metabolic factors outside the nuclei. As such, these...
factors may affect the metabolism of LBM, and not BLM or X-ray, in the production of some DNA-reactive species. Further work, however, is required to fully explain this observation.

The addition of a bulky lipophilic side chain to the N-terminal amine of BLM has produced a novel BLM analogue that, according to the present studies, interacts with DNA in much the same way as do other BLM analogues. Its greater cytotoxic potential than BLM A2 is in part closely associated with its relatively increased lipophilic nature and thus greater uptake into cells. Other explanations for the relative differences in drug cytotoxicity, such as cell repair of drug-mediated DNA lesions, cofactor requirements, or DNA lesion sequence specificity, were considered less likely but cannot be excluded until more studies have been conducted. These data explain the greater efficacy of LBM against the BLM-sensitive human head and neck squamous cell line 183A but do not explain why this particular drug has a greatly reduced fibrogenic potential. Intact animal studies addressing the pharmacokinetics, tissue distribution, and metabolism of LBM are currently under way to address this issue.

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REFERENCES

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