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Comparison of DNA Damage and Single- and Double-Strand Breakage Activities on PM-2 DNA by Talisomycin and Bleomycin Analogs[1]

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ABSTRACT

Single- and double-strand breakage of isolated PM-2 DNA by structural analogs of the glycopeptide antitumor antibiotics bleomycin (BLM) and talisomycin (TLM) was investigated. Breakage of PM-2 DNA was determined by two systems: an ethidium bromide fluorescence assay; and agarose gel electrophoresis. The fluorescence assay, which measures total breakage of DNA including single- and double-strand breakage and alkaline labile damage, showed that the BLM's A_2 and B_2 induced more total DNA breakage than did the TLM's A, B, S_{2b}, and S_{10b}. As measured by the comparison of the concentration of analog required to cause 50% breakage of superhelical DNA, BLM's A_2 and B_2 were 10 times more active than TLM's S_{2b} and S_{10b} and 25 times more active than TLM's A and B. Gel electrophoresis, which measures the extent of both single- and double-strand breakage of DNA, showed that at equivalent levels of breakage of superhelical DNA each of the TLM's caused more double-strand breakage of DNA than did the BLM's. Thus, the structural alterations near the bithiazole in the TLM's, which distinguish them structurally from the BLM's, result in a reduction of the total PM-2 DNA breakage activity and enhanced production of double-strand breaks relative to single-strand breaks by TLM when compared to BLM.

INTRODUCTION

The BLM's[3] are a group of glycopeptide antibiotics, isolated from *Streptomyces verticillus* (27) that have been shown to be effective against a variety of neoplasms (3). The primary mechanism of action of BLM cytotoxicity appears to be interaction with cellular DNA. The effects of BLM on isolated DNA have been shown to include liberation of free bases (5, 18), single-strand breakage (5, 6, 19), double-strand breakage (13, 22), site-specific single- and double-strand breakage (4, 13, 14, 16, 26), noncovalent intermolecular crosslinks (15), and the reduction of DNA melting temperatures (19). BLM also produced breakage of DNA in cells grown in tissue culture, and the extent of degradation was correlated with cell cycle-specific cytotoxicity of the drug (2).

TLM is a new antitumor antibiotic related structurally to BLM. Structures of the 4 TLM analogs used in the studies presented here are shown in Chart 1. They contain 2 new amino acids and a unique sugar, 4-amino-4,6-dideoxy-L-talose that have not been found previously in the BLM complex (11). TLM A has exhibited significantly greater antibiotic activity against a variety of bacteria and fungi than did BLM A_2 (10). Both TLM A and TLM B showed antitumor activity in experimental animal tumor systems (1, 10). However, TLM A was less active than BLM A_2 in *in vitro* DNA breakage systems (24).

The purpose of this study was to further investigate differences in the DNA breakage activities of BLM and TLM by using a number of structural analogs in 2 different *in vitro* analysis systems.

MATERIALS AND METHODS

Analysis of DNA Degradation. A fluorometric assay was utilized to detect strand breakage in covalently closed circular bacteriophage PM-2 DNA. The PM-2 DNA was isolated as described previously (23), which included purification on a cesium chloride/ethidium bromide density gradient and dialysis in 0.15 M NaCl. The analysis was performed by adding the drug, BLM's A_2 or B_2 or TLM's A, B, S_{2b}, or S_{10b} (Bristol Laboratories, Syracuse, N. Y.), to 6 µg PM-2 DNA in a buffer containing 10 mM Tris-HCl (pH 7.5), 20 mM NaCl, and 40 mM DTT in a final volume of 0.120 ml and incubating this mixture at 37° for 30 min. Triplicate experiments were done at each drug concentration. Aliquots of 0.1 ml of the assay solution were placed in 0.9 ml denaturation buffer (0.04 M Na_3PO_4/0.01 M EDTA/0.01 M NaCl, adjusted to pH 12.1 with 0.15 M NaOH) followed by the addition of 0.1 ml ethidium bromide (22 µg/ml in denaturation buffer). Under this alkaline condition, both native linear and single-strand broken circular DNA are denatured. Since at this ionic strength ethidium bromide binds to DNA primarily by intercalation, the dye binds only to native, covalently closed circular DNA and does not bind to broken circular or linear DNA (21). The quantum yield of ethidium bromide fluorescence increases upon intercalation with DNA, and therefore fluorescence greater than background results from the binding of ethidium bromide to covalently closed circular DNA.

Fluorescence of the DNA-drug reaction mixture containing ethidium bromide was determined with an Aminco-Bowman spectrophotofluorometer (American Instrument Co., Silver Spring, Md.) at 530 nm excitation and 590 nm emission. The decrease in concentration of covalently closed circular PM-2 DNA was determined by the percentage of decrease in fluorescence, after subtraction of background fluorescence, relative to control reactions not containing drug. Control reactions were also performed in the absence of DTT, and no decrease in fluorescence was detected because of the addition of DTT. Previous studies (7, 16, 24) have shown that none of the analogs studied compete with ethidium bromide for binding to DNA.

Agarose Gel Electrophoresis. The products of the PM-2

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[3] The abbreviations used are: BLM, bleomycin; TLM, talisomycin; DTT, dithiothreitol; EC_{50}, effective concentration needed to reduce ethidium bromide fluorescence by 50% of control value.

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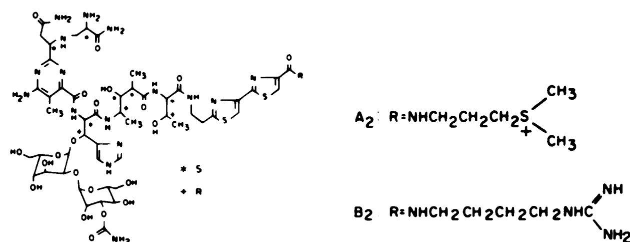
Structure of Bleomycin and Its Analogs



Talisomycin Derivatives

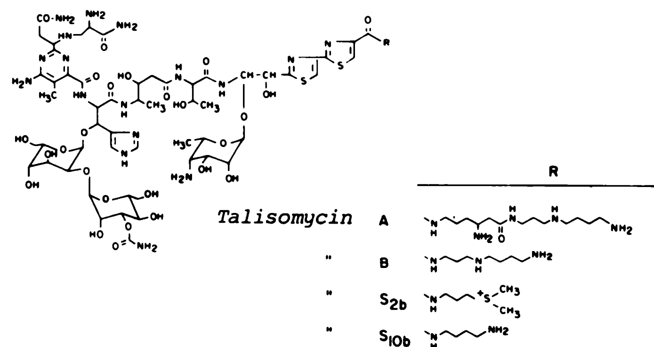


Chart 1. Structures of tested BLM-related drug compounds.

DNA-drug reaction mixtures were electrophoretically separated on agarose gels. The reaction was terminated by the addition of 20 μl of the reaction mixture described above to 20 μl of a solution containing 20 mM EDTA, 70% glycerol, and 0.25% (w/v) bromophenol blue; 20 μl of the resultant mixture were electrophoretically separated in a 1.0% agarose slab gel. Electrophoresis was performed in 0.04 M Tris-HCl, 0.005 M sodium acetate, and 1 mM EDTA (pH 7.8) at 5 V/cm for 10 hr at room temperature. After electrophoresis, the gels were stained in the dark in electrophoresis buffer containing 0.5 μg ethidium bromide per ml for a minimum of 2 hr. 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 (relaxed) circular DNA (Form II).

Quantitation of Forms I, II, and III PM-2 DNA. Quantitative measurements of the amount of DNA that had migrated into the gel were based on the fluorescence enhancement after ethidium bromide intercalation. Following electrophoresis and ethidium bromide incubation, as described above, the slab gels were photographed on a UV plate. The negative films of the gels were scanned with an RFT Model 2955 scanning densitometer (Transidyne General Corporation, Ann Arbor, Mich.), and the relative positions and amounts of each of the DNA forms were recorded. Quantitation of the 3 forms of DNA produced following drug treatment was obtained by measuring the area under the recorded peaks corresponding to the position on the agarose gel for Forms I, II, and III PM-2 DNA as determined by control samples (no drug).

Enhanced fluorescence intensity is expected to be less for the Form I DNA in comparison to an equal mass of Forms II or III DNA, neither of which are topologically restricted in binding of ethidium bromide. In order to estimate the true mass of Form I DNA in a gel band, it was necessary to correct the integrated fluorescence intensity by a multiplicative factor which was determined in the following manner. Three identical samples containing >90% of Form I DNA were subjected to agarose gel electrophoresis, as described above. In different channels on the same gel, 3 identical samples containing the same mass of total DNA as the Form I samples but of Forms II and III (purified on CsCl gradients) were also subjected to electrophoresis. The gel was then stained, photographed, and scanned, and the relative areas of Form I, II, and III peaks were quantitated as described above. From these measurements, a multiplicative factor of 1.44 $\pm$ 0.09 (S.E.), by which the integral fluorescence intensity of Form I PM-2 DNA is reduced by topologically restricted uptake of ethidium bromide as compared to that of Forms II and III DNA, was calculated. This correction factor is consistent with that obtained by others using different methods of analysis (13, 25).

RESULTS

Chart 1 shows the basic structure and the structural modifications of the BLM analogs studied. These compounds include BLM A_2, BLM B_2, TLM A, TLM B, TLM S_{2b}, and TLM S_{10b}. The detailed structural differences of these related compounds will be described as will differences in their ability to cause single- and double-strand breaks in isolated PM-2 DNA.

Degradation of PM-2 DNA by various concentrations of the BLM analogs was compared using the PM-2 DNA fluorescence assay, which detects alkaline-labile damage resulting from nucleotide base release and single- and double-strand breakage of superhelical DNA. The percentage of decrease in ethidium bromide fluorescence, relative to the control, was then plotted *versus* the log of the drug concentration for probit analysis, as shown in Chart 2. Probit analysis permits the accurate quantitation of drug-induced DNA breakage. This analysis linearizes the center region (near the 50% decrease in fluorescence point) of a concentration-percentage of inhibition curve, thereby allowing for a straight line to be fitted by the weighted least-squares method (12). Linear regression analysis was then applied to the probit, and best-fit lines for each analog were constructed. The coefficients of correlation obtained varied from 0.998 to 0.999.

As determined by probit analysis of the fluorescence data (Table 1), approximately equal concentrations of BLM's A_2 and B_2 were required to produce similar decreases in fluorescence with EC_{50} values of 7.74 and 10.7 nM for BLM's A_2 and B_2, respectively.

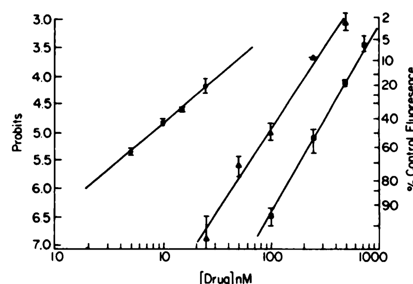


Chart 2. Probit analysis of the percentage of decrease in ethidium bromide fluorescence following incubation with BLM analogs. ●, BLM A_2; ▲, TLM S_{2b}; ■, TLM A. Each *point* represents the average value obtained from 3 separate experiments. *Bars*, S.D. See text for experimental procedures.

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TLM's S_{2b} and S_{10b} were approximately 10-fold less active than BLM's A_2 and B_2 in this DNA breakage system, with EC_{50} values of 95.3 nM and 107 nM, respectively. TLM's A and B were approximately 25-fold less active than the BLM analogs, with EC_{50} values of 263 and 254 nM.

Probit analyses of the fluorescence data show that the slopes of the lines were similar for BLM's A_2 and B_2. The slopes obtained for TLM's A, B, S_{2b}, and S_{10b} were comparable. However, these slopes differed significantly from those of BLM's A_2 and B_2 ($p \ll 0.05$). This difference in slopes suggests differences in PM-2 DNA breakage activity for the BLM analogs relative to the TLM analogs, and may reflect differences in the nature of the mechanism of the total DNA damage induced by these agents.

Treatment of covalently closed circular PM-2 DNA with increasing concentrations of the BLM and TLM analogs resulted in the progressive conversion of Form I DNA to Form II (nicked circular) and Form III (linear duplex) DNA. The 3 topological forms of DNA were resolved by agarose gel electrophoresis and visualized by fluorescence after staining with ethidium bromide (Fig. 1).

At relatively high drug concentrations, a diffuse ethidium bromide staining pattern was observed below the area of migration of Form III DNA in the gels. Visual inspection of the gel demonstrated this in Fig. 1 in the lane corresponding to PM-2 DNA incubated with BLM A_2 at a concentration of 100 nM for 30 min. The diffuse staining pattern was probably the result of nonspecific fragmentation of the linear form DNA (Form III) by the drugs.

Table 1

Probit analysis of PM-2 DNA breakage by BLM analogs as determined by ethidium bromide fluorescence assay

Analog	EC_{50} (nM)	Slope[a]	Correlation coefficient[b]
BLM A_2	7.74	−1.63	−0.999
BLM B_2	10.7	−1.84	−0.994
TLM A	263	−3.31	−0.999
TLM B	253	−3.72	−0.992
TLM S_{2b}	95.3	−3.00	−0.993
TLM S_{10b}	107	−3.05	−0.997

[a] Slope of best-fit line, as determined by linear regression analysis.
[b] Correlation coefficient of best-fit line of probit plot (see text for details).

Fluorescence resulting from this diffuse staining pattern was quantitated and included in calculating the mass fractions of Forms I, II, and III of PM-2 DNA (as described in "Materials and Methods"). This accounts for the difference between a mass fraction of 1.0 and the sum of Forms II and III of DNA produced at high concentrations of BLM A_2 and TLM A, as shown in Charts 3, *A* and *B*.

Control incubations (no drug) in the presence and absence of 40 mM DTT showed no degradation of PM-2 DNA at this concentration of the reducing agent, as determined by agarose gel electrophoresis.

Chart 3*A* shows the mass fractions of each of the forms of PM-2 DNA produced by increasing concentrations of BLM A_2. The decrease in Form I DNA with a corresponding increase in Form II and III DNA's was similar for BLM's A_2 and B_2 at similar concentrations of drug. As shown in Table 2, equivalent concentrations of BLM's A_2 and B_2 resulted in a 50% reduction of Form I DNA and similar production of Form II and III DNA's.

Curves drawn by interpolating between the calculated mass fractions of the 3 forms of DNA produced at increasing concentrations of each of the TLM's were distinctly different from those of the BLM analogs. Chart 3*B* shows the mass fractions of Forms I, II, and III of DNA produced by increasing concentrations of TLM A. As shown in Table 2, the extent of production of Form III DNA relative to Form II DNA at similar reductions of Form I DNA was greater for each of the TLM analogs than for the BLM analogs at the concentrations of BLM A_2 and TLM A which resulted in a 75% reduction of the Form I DNA; the ratios of the increased mass fractions of Form III DNA equaled 0.20 and 1.54, respectively.

The differences in the EC_{50} values noted for the agents in Table 1 as compared to Table 2 reflect differences in the methods used to determine the 50% breakage activity, and experimental variation in the 2 assays. In no case are the differences as large as 1-fold. To avoid problems with interexperimental variations, all compounds were compared simultaneously within each experiment, and all results were tested to determine interexperimental variability. The results and conclusions were statistically valid.

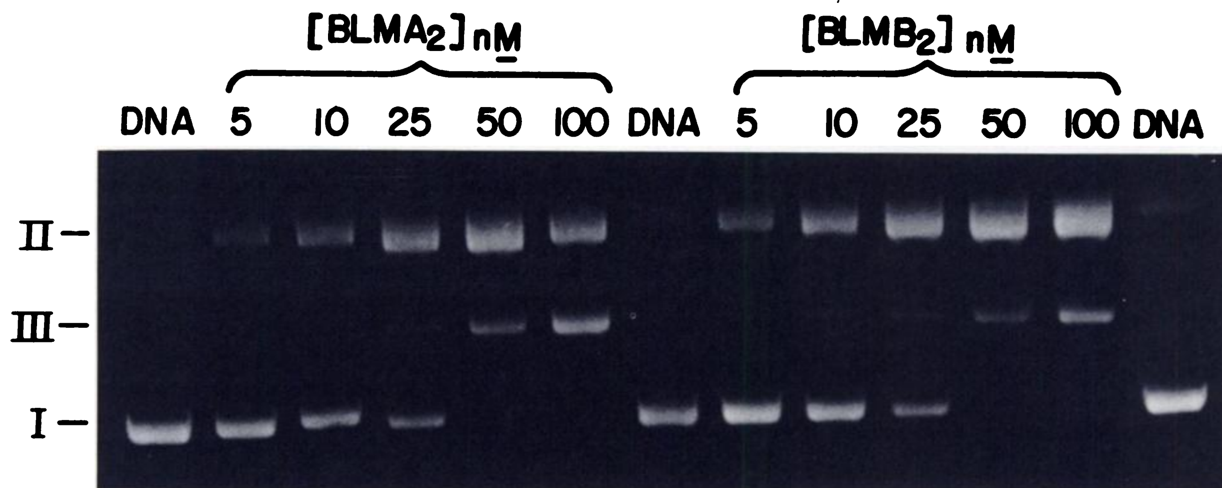

Fig. 1. Agarose gel electrophoresis of PM-2 DNA following incubation with BLM A_2 or B_2 (as described in "Materials and Methods"). *Numbers* on *left*, area of migration of: single-strand broken (relaxed) DNA (Form II); double-strand broken (linear) DNA (Form III); and covalently closed superhelical (circular) DNA (Form I). *Top*, the analog and its concentration in the reaction mixture. *DNA lanes*, control (no drug).

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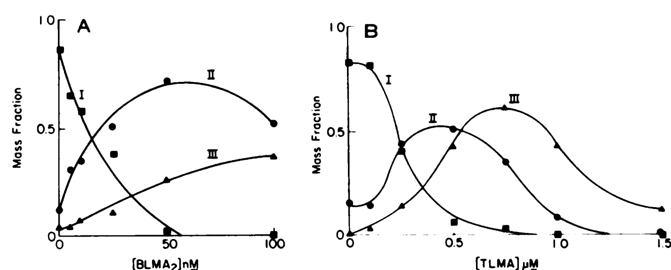

Chart 3. Effect of increasing concentrations of BLM or TLM on PM-2 DNA. Mass fraction of DNA in each form as a function of increasing concentrations of BLM A_2 (*A*) and TLM A (*B*).

Table 2
Ratios of mass fractions of Form III DNA/Form II DNA at 50% reductions of Form I PM2 DNA

Analog	Concentration (nM)[a]	Form III/Form II DNA[b]
BLM A_2	17	0.19
BLM B_2	19	0.16
TLM A	250	0.45
TLM B	200	0.69
TLM S_{2b}	170	0.45
TLM S_{10b}	150	0.68

[a] Concentration of analog required to reduce the control level of the Form I DNA mass fraction by 50 as determined by agarose gel electrophoresis as described in "Materials and Methods."
[b] Ratio of the mass fractions of Form III/Form II PM2 DNA. Mass fractions were measured by subtracting control levels of Forms II or III DNA from mass fraction produced at indicated analog concentrations.

DISCUSSION

Like BLM, TLM produces strand breaks in isolated and intracellular DNA (24). BLM- and TLM-induced double-strand breakage may contribute to their cytotoxicity. Cytological studies using cultured mouse fibroblasts showed that fragmentation of metaphase chromosomes occurred when cells were incubated with high concentrations of BLM (20). Other studies suggested that cytotoxicity correlated with the degree of DNA degradation induced by BLM (8, 17). Thus, differences between BLM and TLM in both the type of DNA breakage activities and the mechanisms involved in DNA breakage may account for the greater antibiotic activity of TLM against a variety of bacteria and fungi and the different levels of antitumor activities of the 2 drugs in experimental tumor systems (10).

It has been reported previously, using several different assay systems, that BLM A_2 had a greater DNA breakage activity against both intracellular and isolated DNA than did TLM A (24). We have extended these findings in the present study using 4 additional BLM analogs. These results indicate that in terms of total PM-2 DNA breakage, as measured by the alkaline ethidium bromide fluorescence assay, BLM's A_2 and B_2 were approximately 10 times more active than TLM's S_{2b} and S_{10b} and 25 times more active than TLM's A and B. These data suggest that structural alterations near the bithiazole of BLM, such as those present in the TLM's, reduce the potency for total DNA damage. This is directly evidenced by the 10-fold decrease in total DNA damage activity of TLM S_{2b} when compared to that of BLM A_2 (Table 1), inasmuch as the structures of these 2 analogs differ only in the 4,6-dideoxy-L-talose sugar and 2 amino acids near the bithiazole present in TLM S_{2b} but absent in BLM A_2.

It has recently been reported that the positive charge at the terminal amine facilitates the binding of BLM's to nucleic acids (7, 9), as well as affecting their ability to break DNA.[4] Under the conditions used in our experiments, the COOH-terminal amine of TLM's S_{2b} (3-aminopropyldimethylsulfonium) and S_{10b} (putresine) confers a charge of positive one on these analogs. The COOH-terminal amines of TLM's A and B give these 2 analogs charges of +2 and +3, respectively. The ethidium bromide fluorescence assay (Table 1) and the agarose gel assay (Table 2) demonstrate that TLM's S_{2b} and S_{10b} are more potent DNA damaging agents than are TLM's A and B. Thus, within this group of TLM analogs, a decrease in the charge of the molecule from positive +3 and +2 to a charge of +1 appears to result in an increase in total DNA damage activity. These data are consistent with data reported for BLM analogs of varying COOH-terminal amine charge[4] and the results shown for BLM's A_2 and B_2, which have COOH-terminal amines with a charge of +1 and demonstrate similar breakage activity (Tables 1 and 2). Previous studies (7, 24) have shown that there is no direct correlation between DNA-binding constants and the extent of DNA degradation. Rather, the rate-limiting step appears to be the oxidation of Fe(II) and consequent generation of free radicals. Thus, it is not surprising that analogs with a greater positive charge, and thus probably a higher affinity for DNA, may not be more potent DNA degradative agents.

Quantitation of direct single- and double-strand breakage of DNA by agarose gel electrophoresis demonstrates a difference in the relative amounts of production of Form II and III DNA's from Form I DNA for the BLM analogs as compared to the TLM analogs (Table 2; Chart 3, *A* and *B*). It has been demonstrated that the production of Form III PM-2 DNA by BLM cannot be the result of the accumulation of random single-strand breaks and, therefore, represents direct double-strand breakage of DNA by the drug (5). Therefore, the more extensive production of Form III DNA by the TLM's than by the BLM's suggests that interaction of the TLM's with DNA results in more double-strand breaks per molecule of Form I PM-2 DNA degraded than that resulting from the BLM analogs.

The differences in the ratios of the single- and double-strand DNA breakage activities of the TLM's and BLM's suggest the importance of the structural modifications between these 2 structurally related molecules in determining the types of DNA breakage induced by the drugs. Thus, the talose sugar and the 2 amino acids located near the bithiazole in TLM appear to be important in conferring the preferential double-strand breakage activity, relative to single-strand breakage activity, to the TLM analogs.

In summary, our results indicate that structural and/or conformational alterations near the bithiazole of the bleomycins (*e.g.*, TLM) affect both the total DNA damage activity and the ability to produce double-strand breaks on PM-2 DNA. We are currently undertaking experiments to further investigate the differences in single- and double-strand breakage activities of the BLM's and the TLM's in terms of other structural modifications and charge differences of the drug molecules and the topological nature of the DNA substrate. These results in combination with data concerning the apparent differences in site

[4] C. H. Huang, C. K. Mirabelli, Y. Jan, and S. T. Crooke. Single-strand and double-strand DNA breaks produced by several bleomycin analogs, submitted for publication.

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specificities of double-stranded DNA fragmentation[5] (16) and cell cycle specificities[6] for TLM and BLM analogs may suggest molecular and cytological explanations for their different antibacterial and antitumor activity. With this information, it may be possible to design new BLM analogs which cleave at specific sites in cellular DNA, resulting in relatively more selective cytotoxic activities.

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