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Single-Strand and Double-Strand Deoxyribonucleic Acid Breaks Produced by Several Bleomycin Analogues†

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ABSTRACT: Production of single-strand breaks (ssb) and double-strand breaks (dsb) of PM2 phage DNA by several structurally related bleomycin (BLM) analogues was studied by gel electrophoresis. BLM A_2 and BLM B_2 produced a comparable extent of dsb. In various experiments, BLM A_2 and BLM B_2, at 22–41 ng/mL, degraded 50% of the form I DNA into 33–38% form II and 12–17% form III DNA. BLM B_1' produced ssb and dsb at a ratio similar to that of BLM A_2, but both at a rate less than half that of BLM A_2. Phleomycin (PLM) D_1 induced an equivalent amount of ssb but only one-eighth of dsb induced by BLM B_2. The relatively lower extent of dsb production for PLM D_1 was observed either in borate buffer (pH 9.5) or in Tris-HCl buffer (pH 7.5) and in the presence or absence of exogenous Fe(II). Deamido-BLM A_2 produced ssb to an extent approximately half that of BLM A_2 and dsb to less than one-eighth that of BLM A_2. The following conclusions were drawn. (1) BLM analogues produced ssb and dsb to different extents and ratios. (2) The ratio of dsb to ssb varied depending on the analogue, indicating a lack of a direct correlation between ssb and dsb. (3) The extent of ssb and dsb was affected by modifications on both the C- and N-terminal half-molecules of BLM: modification of either the N-terminal amide or the bithiazole greatly reduced dsb, whereas changes in structure or charge in the C-terminal amine affected ssb and dsb to a similar extent.

The bleomycins (BLM's)[1] are a family of glycopeptide antitumor antibiotics which were isolated by Umezawa et al. (1966) and clinically used for treatment of certain human cancers (Crooke & Bradner, 1976). The antitumor activity is thought to be related to the ability of bleomycins to degrade DNA. In vitro, BLM-induced DNA breakage has been suggested to involve the complex formation of bleomycin, Fe(II), and oxygen, with the subsequent production of free radicals which cause DNA strand breaks (Sausville et al., 1978; Lown & Sim, 1977). For the drug–Fe(II) complex formation, several models have been proposed (Dabrowiak et al., 1978; Takita et al., 1978; Oppenheimer et al., 1979). The generation of free radicals from drug–Fe(II) complexes has been observed (Lown et al., 1978; Sugiura & Kikuchi, 1978; Oberley & Buettner, 1979). Both the bithiazole and the C-terminal amine moieties were suggested to interact with DNA (Chien et al., 1977), and the possible intercalation of the bithiazole moiety was proposed to provide the observed G–C, G–T, and T–A base-sequence specificity of breakage (Takeshita et al., 1978). Nevertheless, the precise mechanisms of DNA–drug interaction and the DNA breakage remain to be elucidated (Lloyd et al., 1978a,b).

Recent studies by Kasai et al. (1978) and Huang et al. (1980) using fluorescence quenching techniques suggested variations in the mode of DNA–drug interaction among BLM analogues active in degrading DNA. It is thus possible that different BLM analogues may produce different types of DNA damage as a result of variation in the mode of interaction with DNA. In this report, we have characterized and quantitated by gel electrophoretic techniques the extent of production of single-strand breaks (ssb) as well as double-strand breaks (dsb) of PM2 phage DNA by various structurally related BLM analogues. Results from this study indicate some structure–activity relationships with mechanistic implications.

Materials and Methods

Chemicals. Bleomycin A_2, bleomycin B_2, bleomycin B_1', and phleomycin D_1 were obtained from the Bristol Laboratories, Syracuse, NY. Deamidobleomycin A_2 was a generous gift from Professor H. Umezawa, Institute of Microbial Chemistry, Tokyo, Japan. PM2 DNA was isolated according to the procedure of Strong & Hewitt (1975). Usually, PM2 DNA preparations containing >84% of superhelical form I DNA were used. Tris, borate, Na_2EDTA, β-mercaptoethanol, and ethidium bromide were obtained from Sigma Chemical Co., St. Louis, MO, agarose-ME was obtained from Miles Laboratories, Elkhart, IN, and $FeCl_2$ was from Mallinckrodt Co., St. Louis, MO.

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[1] Abbreviations used: BLM, bleomycin; PLM, phleomycin; Tris, tris(hydroxymethyl)aminomethane; Na_2EDTA, disodium ethylenediaminetetraacetic acid; EB, ethidium bromide; DNA, deoxyribonucleic acid.

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Assays of DNA-Breakage Activity of Bleomycin Analogues. For DNA-breakage activity of bleomycin analogues a reaction mixture (final volume, 0.5 mL) was used which contained 50 mM borate buffer (pH 9.5) or 50 mM Tris-HCl (pH 7.5), 25 mM β-mercaptoethanol, 112–121 μM PM2 form I DNA preparations, and varying amounts of bleomycin analogues ranging from 0 to 2 μg/mL. The reactions were performed at room temperature for 30 or 60 min and were terminated with the addition of an equal volume of a dye–EDTA mixture containing 56% glycerol (v/v), 50 mM EDTA, and 0.05% bromophenol blue (w/v). Aliquots were then taken and assayed for the production of ssb and dsb by agarose gel electrophoresis as described below.

Agarose Gel Electrophoresis. Aliquots of the reaction mixtures which contained 0.8–1.4 μg of DNA were layered onto 0.9% agarose slab gel and electrophoresed in a horizontal, slab gel apparatus for 6–8 h at room temperature, with a 40 mM Tris-HCl buffer containing 5 mM sodium acetate and 1 mM EDTA, pH 7.8. After electrophoresis, gels were stained with 0.5 μg/mL ethidium bromide (EB) in the electrophoresis buffer for at least 2 h. The stained gels were then excited with a transilluminator (Ultra-violet Products, Inc.) and photographed with a polaroid CU-5 Land Camera equipped with a No. 8 Kodak Wratten gelatin filter (Eastman Kodak Co., Rochester, NY) and type 665 Land films. The negative films of gels were used for densitometric scannings.

Quantitation of Single-Strand and Double-Strand DNA Breaks by Densitometric Scannings of Negative Films of Gels. The negative films of the EB-stained patterns of the drug-treated PM2 DNA were scanned with a recording Transidyne General Densitometer equipped with an automatic computing integrator. The production of the nicked, relaxed, duplex form II DNA and the linear form III DNA from the covalently closed, superhelical form I was considered to be the result of single-strand and double-strand breaks, respectively. The relative stainability of the form I, form II, and form III DNA in the agarose gels was determined by using known amounts of respective DNA preparations. In agreement with previous reports (Lloyd et al., 1978a,b) it was found that at the same amount of DNA, the EB fluorescence intensity of form I DNA was only 70% that of form II or form III DNA, and thus this factor was used to normalize all observations. In agreement with the report of Prunell et al. (1977), at the DNA concentration range used in the present study, the densitometric readings were linearly proportional to the DNA concentrations.

Results

Structure of Tested Bleomycin Analogues. Figure 1 shows the structures of bleomycin (BLM) A_2, BLM B_2, BLM B_1', deamido-BLM A_2, and phleomycin (PLM) D_1. All BLM analogues except PLM D_1 have an aromatic, coplanar bithiazole moiety. In PLM molecules, one of the bithiazole rings is hydrogenated and thus the coplanar property of the bithiazole moiety is destroyed. BLM A_2 has a (3-aminopropyl)dimethylsulfonium group as the C-terminal amine, which carries a positive charge at near neutral pH. Both BLM B_2 and PLM D_1 have the same C-terminal amine, agmatine, which also is positively charged. BLM B_1' has a simple C-terminal amine, $-NH_2$, which bears no charge. Deamido-BLM A_2, discovered by Umezawa et al. (1974) is the product of the hydrolyic deamination of BLM A_2, with the removal of the N-terminal amide group.

DNA Breaks Produced by BLM A_2 and PLM D_1. Figure 2 shows the EB-stained DNA banding pattern in the agarose gels after treatment of PM2 phage form I DNA with BLM A_2 or PLM D_1 for 30 min (Figure 2A) or 60 min (Figure 2B)

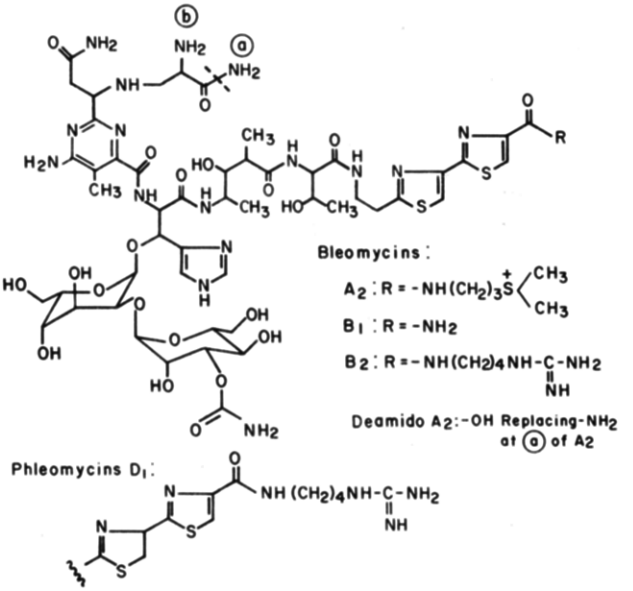

FIGURE 1: Structure of tested bleomycin analogues.

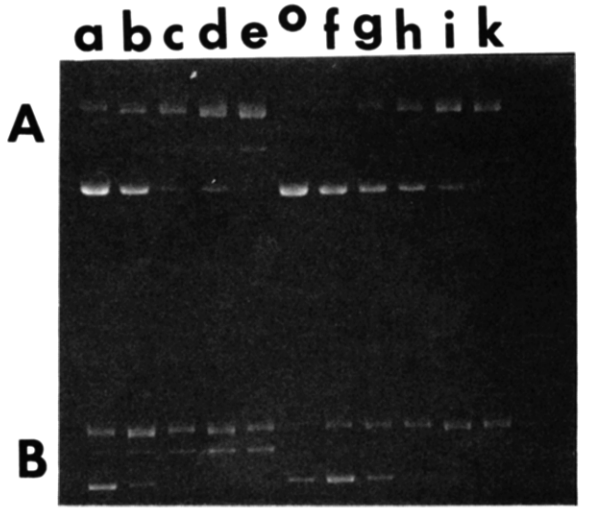

FIGURE 2: Agarose gel electrophoretic pattern of ethidium bromide strained PM2 DNA after treatment with BLM A_2 (lanes a–e) and PLM D_1 (lanes f–k) for 30 (A) and 60 (B) min at room temperature. Reaction mixture contained 50 mM borate buffer (pH 9.5), 37.6 μg/mL (0.114 μM) PM2 DNA, and increasing amounts of bleomycin analogues. (Lane 0) Untreated DNA. (Lanes a–e) 10, 20, 30, 50, and 80 ng/mL BLM A_2, respectively. (Lanes f–k) 10, 20, 30, 50, and 80 ng/mL PLM D_1, respectively. For lane 0 in panel B, only one-fourth the amount of DNA was applied to the gel. 1 nM BLM analogue is equivalent to 1.45 ng/mL. Agarose gel at 0.9% was used, and DNA migrated from top to bottom in the order of decreasing distance of form I, form III, and form II DNA.

in 50 mM borate buffer, pH 9.5, a condition found to be optimal for the DNA breakage activity of BLM A_2. In Figure 2A, lane 0 represents the untreated PM2 DNA preparation, which gave a major band corresponding to the superhelical, covalently closed, duplex form I DNA and a weak band corresponding to the nicked, relaxed, circular duplex form II DNA. A single-strand break (ssb) changes form I DNA to form II DNA. A double-strand break (dsb), produced either directly or as a result of two closely-spaced single-strand breaks at complementary strands, changes either the form I or form II DNA into the linear, open duplex form III DNA. Under our electrophoretic conditions, form III DNA migrates to a position between that of form I and form II DNA (Johnson & Grossman, 1977). Lanes a–e show that treatment of form I DNA with BLM A_2 at increasing concentrations caused a

Table I: Percentage Distribution of DNA after Treatment of BLM Analogues to Cause 50% and 75% Reduction of Form I DNA

		at 50% reduction			at 75% reduction		
	BLM analogues	drug concn (ng/mL)	II (%)	III (%)	drug concn (ng/mL)	II (%)	III (%)
borate, pH 9.5	A_2	25	38	12	56	56	19
	B_2	25	39	11	58	57	20
	A_2	41	35	16			
	B_1'	120	41	10			
	A_2	22	34	16	50	51	25
	deamido A_2	46	45	5	124	66	7
	B_2	30	33	17	62	50	25
	PLM	44	46	2	84	73	3
	B_2 + Fe(II)	15	38	13	27	57	17
	PLM + Fe(II)	20	47	2	45	69	4
Tris-HCl, pH 7.5	A_2	140	36	12	340	57	19
	deamido A_2	280	48	3	460	68	6
	B_2	110	39	11	250	57	19
	PLM	250	48	3	320	69	7

gradual decrease in the banding intensity of form I DNA and a simultaneous increase in that of both the form II and form III DNA, demonstrating the occurrence of both ssb and dsb. Lanes f–k show the results of PLM D_1 treatment at increasing concentrations. The decrease in form I DNA was accompanied by an increase in form II DNA. However, less form III DNA was produced by PLM D_1 than by BLM A_2. Results of gel patterns after a 60-min incubation of DNA (Figure 2B) demonstrated similar DNA breakage patterns for both drugs although the DNA degradation was more extensive than at 30 min.

Quantitative Analysis of DNA Breaks Produced by BLM A_2 and PLM D_1. Quantitative analysis by densitometry of the ssb and dsb production by BLM A_2 and PLM D_1 treatment for 30 min, as shown in Figure 2A, was performed, and the results are shown in Figure 3. The untreated PM2 DNA preparation used in this experiment contained approximately 93% form I DNA and 7% form II DNA. Form III DNA was virtually undetectable. The results demonstrate that at equivalent concentrations the extent of production of form II DNA by the two drugs was similar. However, BLM A_2 produced ~50% as much form III DNA as form II DNA, whereas PLM D_1 produced very little form III DNA. As shown in Table I, a 50% reduction of form I DNA occurred at 30 ng/mL BLM A_2 and at 44 ng/mL PLM D_1. A 50% reduction of form I DNA resulted in 33% form II DNA and 17% form III DNA for BLM A_2 and 46% form II DNA and 2% form III DNA for PLM D_1. A 75% reduction of form I DNA resulted in 50% form II and 25% form III DNA for BLM A_2 but 73% form II and 3% form III for PLM D_1. Thus, although BLM A_2 and PLM D_1 produced ssb to a similar extent, PLM D_1 produced very few dsb.

DNA Breaks Produced by BLM B_2. BLM B_2, like PLM D_1, has a positively charged (+1) agmatine group as the C-terminal amine. As shown in Table I, a 50% reduction of form I resulted in 39% form II and 11% form III for BLM B_2 at 25 ng/mL and in 38% form II and 12% form III DNA for BLM A_2 at the same concentration. Similar types of distribution of DNA isomers were seen for both drugs at concentrations which resulted in a 75% loss of form I DNA. Thus, the differences in the C-terminal amine between BLM B_2 and BLM A_2 do not significantly affect the extent and the types of DNA breakage. These results also suggest that the differences in the production of form III DNA between PLM D_1 and BLM A_2 are not due to the difference in the C-terminal amine, but to the hydrogenation of one of the thiazole rings.

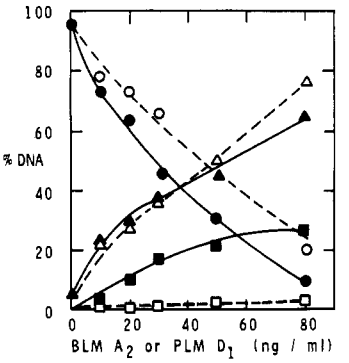

FIGURE 3: Percentage distribution of DNA conformational isomers after treatment with BLM A_2 or PLM D_1 in 50 mM borate buffer (pH 9.5) for 30 min. Data were obtained from the gel pattern shown in panel A, Figure 2. For BLM A_2 treatment: (●) form I DNA; (▲) form II DNA; (■) form III DNA. For PLM D_1: (○) form I DNA; (△) form II DNA; (□) form III DNA.

DNA Breaks Produced by BLM B_2 or PLM D_1 in the Presence of Fe(II). Inasmuch as the degradation of DNA in the absence of exogenous Fe(II) is thought to be due to oxidation of factitious Fe(II) which is difficult to control, we have studied the effects of these agents in the presence of varying concentrations of Fe(II) to prove that differences in single- and double-strand breakage are not simply due to slightly different cofactor requirements. In the presence of exogenous Fe(II), PLM D_1 produced form II DNA to an extent similar to that produced by BLM B_2, but PLM D_1 still produced very few form III DNA molecules. As shown in Table I, a 50% reduction of form I DNA resulted in 38% form II and 13% form III DNA for BLM B_2 and 47% form II and 2% form III for PLM D_1. Similar results were observed at concentrations degrading 75% of form I. Thus, external Fe(II) stimulated the production of ssb by BLM B_2 and PLM D_1 in a similar manner but failed to induce any significant production of dsb by PLM D_1.

DNA Breakage Produced by BLM B_2 and PLM D_1 in Tris-HCl, pH 7.5. In Tris-HCl buffer, pH 7.5, the overall DNA degradative activity of both BLM B_2 and PLM D_1, as measured from the extent of the disappearance of form I DNA, was less than that observed in the borate buffer, pH 9.5. Furthermore, in contrast to the equivalent potency observed in borate buffer at pH 9.5, the potency of BLM B_2 was almost twice that of PLM D_1. As shown in Table I for BLM

B_2, degradation of 50% of form I DNA resulted in the production of 39% form II and 11% form III DNA, whereas PLM D_1 resulted in the production of 48% form II and 3% form III DNA. A reduction of 75% form I resulted in 57% form II and 19% form III DNA for BLM B_2 and 69% form II and 7% form III for PLM D_1. Thus, although the extent of DNA degradation by both drugs was less in Tris-HCl buffer at pH 7.5, PLM D_1 still produced fewer dsb than BLM B_2. BLM A_2 (results not shown) behaved in a manner equivalent to that of BLM B_2.

DNA Breakage Induced by BLM B_1'. The C-terminal amine of BLM B_1', $-NH_2$, is not charged under the conditions used in the present study. Table I shows that treatment with increasing concentrations of BLM B_1' produced form II and form III DNA in a manner similar to that induced by BLM A_2 treatment, but only at ~3 times the concentrations. Table I indicates that a 50% reduction of form I DNA resulted in 41% form II and 10% form III DNA for BLM B_1' and 35% form II and 16% form III for BLM A_2 under the same conditions. Thus, since the form II to form III DNA ratios produced by both drugs were similar, neither the lack of charge nor the shortening of the C-terminal amine (or both) reduced the extent of form III DNA production relative to the production of form II DNA.

DNA Breaks Produced by Deamido-BLM A_2 in Borate Buffer at pH 9.5. Figure 4A shows the quantitative analyses of the DNA breaks after deamido-BLM A_2 treatment in the borate buffer (pH 9.5). The results of BLM A_2 treatment in the same experiment are also shown as a reference. These results clearly demonstrate that the deamidation of the N-terminal amide moiety of the BLM A_2 molecule significantly reduced the potency for degradation of form I DNA in borate buffer at pH 9.5, being approximately half that observed for BLM A_2. The extent of production of form II DNA by deamido-BLM A_2 was ~50% that of BLM A_2, whereas deamido-BLM A_2 produced from III to an extent only one-sixth to one-eighth that produced by BLM A_2. As shown in Table I, a 50% reduction of form I DNA induced by deamido-BLM A_2 resulted in 45% form II and 5% form III DNA but 34% form II and 16% form III DNA when induced by BLM A_2 in the same experiment. At a 75% reduction of form I DNA, deamido-BLM A_2 produced 7% form III whereas BLM A_2 produced 25% form III DNA. Thus, at concentrations inducing equivalent degradation of form I DNA, deamido-BLM A_2 produced much lower amounts of form III DNA than did BLM A_2, i.e., fewer double-strand breaks.

DNA Breaks Produced by Deamido-BLM A_2 in Tris-HCl at pH 7.5. Figure 4B shows the DNA-breakage activity of deamido-BLM A_2 and BLM A_2 in Tris-HCl at pH 7.5. The DNA degrading activity of both drugs was reduced, being only one-seventh that obtained in borate buffer. However, at concentrations resulting in degradation of equivalent amounts of form I DNA, desamido-BLM A_2 still produced much less form III DNA than did BLM A_2. Table I shows the percentage distribution of form II and form III DNA produced by desamido-BLM A_2 and BLM A_2 at concentrations which resulted in a reduction of 50% and 75% of form I DNA. Equivalent ratios of form II to form III DNA were obtained in the presence of absence of exogenous Fe(II). Addition of Fe(II) resulted in enhanced DNA degradative activity for both compounds but did not alter the ratio of dsb to ssb for either drug (results not shown).

Discussion

The production of single-strand breaks and double-strand breaks in PM2 superhelical form I DNA by treatment with bleomycin can be accurately measured by gel electrophoresis of the form II and form III DNA, respectively (Lloyd et al., 1978a,b; Johnson & Grossman, 1977; Strong & Crooke, 1978). Only the first single-strand break in each DNA molecule is detected since a single break is sufficient to relax the superhelical DNA to form II DNA. The production of the double-strand breaks can be a result of direct double-strand breaks which occurred as a single event or of two closely spaced single-strand breaks which occurred in complementary strands within the "DNA breathing distance". Previous studies (Povirk et al., 1977) indicated that most double-strand DNA breaks produced by the clinical preparation of bleomycins did not result from accumulation of random single-strand breaks.

As summarized in Table I, the removal of the N-terminal amide (deamido-BLM A_2) or the modification of the bithiazole moiety (PLM D_1) but not changes in the C-terminal amine (e.g., BLM B_1') resulted in a marked reduction of the compounds' ability to induce dsb in PM2 DNA. Both the bithiazole and the C-terminal amine have been reported to interact with DNA (Chien et al., 1977). The C-terminal amine has been suggested to interact ionically. Consequently, since changes in the C-terminal amine resulted in alterations in the potency (e.g., BLM B_1' vs. BLM A_2) for production of DNA damage but did not affect the ratio of double-strand to sin-

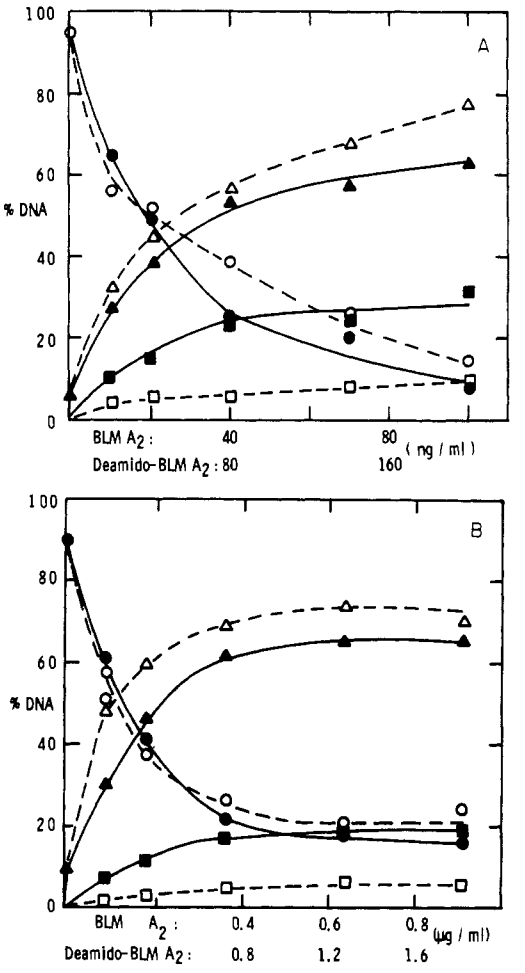

FIGURE 4: (A) Percentage DNA distribution after treatment of 114 μM DNA with BLM A_2 or deamido-BLM A_2 in 50 mM borate buffer (pH 9.5) for 30 min. For BLM A_2 treatment: (●) form I DNA; (▲) form II DNA; (■) form III DNA. For deamido-BLM A_2 treatment: (○) form I DNA; (△) form II DNA; (□) form III DNA. (B) Percentage DNA distribution after treatment of 119 μM DNA with BLM A_2 or deamido-BLM A_2 in 50 mM Tris-HCl buffer (pH 7.5) for 30 min. For BLM A_2 treatment: (●) form I DNA; (▲) form II DNA; (■) form III DNA. For deamido-BLM A_2 treatment: (○) form I DNA; (△) form II; (□) form III DNA.

gle-strand breakage activity, we conclude that the C-terminal amine is involved nonspecifically in DNA binding. However, ionic interactions between BLM analogues and DNA do not selectively affect dsb activity.

In contrast, the bithiazole portion of the BLM molecule has been shown to bind to DNA nonionically, and perhaps via an intercalative-type interaction (Murakami et al., 1976; Povirk et al., 1979). Moreover, it has been proposed that the binding of the bithiazole to DNA may provide the basis for the observed preference for cleavage of G–C, G–T, and T–A sequences in DNA (D'Andrea & Haseltine, 1978; Takeshita et al., 1978). The results of the present study suggest that the proper interaction between the bithiazole moiety and DNA is essential for the production of dsb but is not required for the induction of ssb since PLM D_1 induced almost no dsb but was as potent as BLM B_2 in induction of DNA damage which was manifested as ssb. This could reflect a generic alteration in the binding of these molecules to DNA or a selective reduction in the ability of PLM D_1 to interact productively with sites in PM2 DNA susceptible to double-strand cleavage.

In any event, the results of the present study extend the observations that the phleomycins interact with DNA in a manner distinctly different from the bleomycins. For example, PLM has been reported to induced circular dichroism effects which differ from those produced by BLM (Krueger et al., 1973), and PLM increased whereas BLM decreased the melting temperature of DNA (Nagai et al., 1969). Furthermore, the binding of PLM to DNA was prevented by blocking with Hg(II) at the 2-carbonyl of thymine in DNA (Pietsch & Garrett, 1968), but that of BLM was unaffected (Muller et al., 1972). Moreover, we have observed (C. H. Huang and S. T. Crooke, unpublished observations) that the fluorescence of the 4-aminopyrimidine moiety of PLM D_1 was markedly enhanced when PLM D_1 interacted with DNA. Such a phenomenon was not observed with any BLM analogue. Thus, a variety of lines of evidence suggest that the interaction of PLM analogues with DNA differs from the interaction of BLM Analogues, and this difference clearly should be investigated in more detail.

The demonstration that deamido-BLM A_2 was less potent in degrading PM2 DNA than was BLM A_2 qualitatively confirms previously reported results (Umezawa, 1976). The selective loss of potency for induction of dsb may account for the loss of potency for induction of DNA total damage. A potential explanation for these effects, particularly the selective loss of dsb induction potency, is unclear.

The N-terminal amide group bears little charge, and thus little ionic interaction between this group and DNA is expected. However, this portion of the molecule is involved in interactions with cations (Takita et al., 1978; Dabrowiak et al., 1978). The removal of this amide group resulted in the change in pK value of the neighboring α-amino group of the β-aminoalanine moiety from 7.3 to 9.3 (Umezawa, 1976). It is possible that the pK change affects the mode of the coordination of the α-amino group with Fe(II). A proper conformation of the coordination complex of Fe(II) with bleomycin, especially the ligation at the α-amino site, may induce specific conformational changes favoring the subsequent oxygen ligation and DNA breakage (Huang et al., 1979; Sugiura et al., 1979; Oppenheimer et al., 1979). Therefore, it is possible that deamination as in deamido-BLM A_2 may alter the proper conformational changes by affecting the mode of BLM–Fe(II) coordination and thus may change the extent and the type of the DNA breaks. The conformational aspects of the deamination are currently under study. Other possible explanations of the effect of N-terminal deamination, such as the alteration of the possible hydrogen bonding between the amide and DNA or alterations in the ability to recycle Fe(II), cannot be excluded (Sugiura et al., 1979).

The extent of DNA degradation and the ratio of dsb and ssb for BLM A_2 and BLM B_2 at two pH values and in the presence or absence of exogenous Fe(II) were very similar, although the rate of ssb and dsb produced by both drugs was slower in Tris-HCl buffer (pH 7.5), as compared to that in borate (pH 9.5). The C-terminal amine moiety of both drugs bears one positive charge under the current conditions and is similar in length. Thus, it is possible that the changes in either the charge or the length, or both, of the terminal amine may affect the potency for inducing DNA breakage but not the production ratio of the form II and form III DNA.

The observation that bleomycin analogues induced different rates and types of DNA breaks, depending on different structural modifications may provide opportunities for futher design and development of new bleomycin analogues directed to enhance or reduce specific types of DNA damage. It is conceivable that the double-strand breaks as compared to single-strand nicking might be less amenable to repair, although conclusive evidence has yet to be presented (Miyaki et al., 1973). Thus, it may be possible to design analogues which produce only ssb under certain conditions and dsb under other conditions. Perhaps such analogues might produce more selective cytotoxicity, for example by producing dsb only in cells with a lower pH. Clearly, the results presented in this paper and results showing that talisomycin analogues have site specificities for dsb different from BLM analgoues (Mirabelli et al., 1979) provide an initial basis for the rational design of new BLM analogues.

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Synthesis by DNA Polymerase I on Bleomycin-Treated Deoxyribonucleic Acid: A Requirement for Exonuclease III†

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ABSTRACT: ΦX174 RFI DNA treated with bleomycin (BLM) under conditions permitting nicking does not serve as a template-primer for *Escherichia coli* DNA polymerase I. Purified exonuclease III from *E. coli* and extracts from wild-type *E. coli* strains are able to convert the BLM-treated DNA to suitable template-primer, but extracts from exonuclease III deficient strains are not. Brief digestion by exonuclease III is enough to create the template-primer, suggesting that the exonuclease III is converting the BLM-treated DNA by a modification of 3′ termini. The exonucleolytic rather than the phosphatase activity of exonuclease III appears to be involved in the conversion. Comparative studies with micrococcal nuclease indicate that BLM-created nicks do not have a simple 3′-P structure. Bacterial alkaline phosphatase does not convert BLM-treated DNA to template-primer. The endonuclease VI activity associated with exonuclease III does not incise DNA treated with BLM under conditions not allowing nicking, in contrast to DNA with apurinic sites made by acid treatment, arguing that conversion does not require the endonuclease VI action on uncleaved sites.

Bleomycin (BLM),[1] a glycopeptide antibiotic produced by *Streptomyces verticillus* (Umezawa et al., 1966), is used therapeutically on certain types of tumors. The main target of the drug is DNA (Muller & Zahn, 1977). BLM can produce loss of bases from DNA, alkali-sensitive sites, and under appropriate conditions strand scissions (Haidle et al., 1972; Muller & Zahn, 1977; Ross & Moses, 1978; Sausville et al., 1978a). Analyses of the products (oligonucleotides) of BLM-treated DNA have shown that BLM may create heterogeneous breakdown products in respect to the terminal structures (Sausville et al., 1978; D'Andrea & Haseltine, 1978). Lack of 3′-P at the nicked sites has been suggested since inorganic phosphate is not released by exonuclease III (Kuo & Haidle, 1973; Kappen & Goldberg, 1978).

Apparently due to the combination of strand scissions and sites of base loss, BLM causes inhibition of DNA synthesis both in vivo and in vitro (Yamaki et al., 1971; Kuo & Haidle, 1973; Muller & Zahn, 1977; Kappen & Goldberg, 1978). However, little is known about the reactivity of BLM-produced lesions in the DNA molecule or repair reactions of cells exposed to this drug. On the basis of the above data, it appears reasonable that the cellular response to BLM involves steps of the excision repair process, differing perhaps in the early events, depending on whether the damaged nucleotides must be excised or not. We have studied the response to BLM in permeable *Escherichia coli* (Ross & Moses, 1977) and found that the agent stimulates nonconservative repair synthesis, but does not inhibit semiconservative synthesis, in contrast to ultraviolet (UV) irradiation. Thus, BLM appears to be a useful probe of cellular DNA repair processes with some features differing from UV irradiation.

To understand the cellular repair synthesis in response to BLM and to analyze the requirements for such repair synthesis, we have investigated the ability of DNA polymerase

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[1] Abbreviations used: BLM, bleomycin; RFI, covalently closed circular replicative form I DNA; dNTP, deoxynucleoside triphosphate; DTT, dithiothreitol; Tris, tris(hydroxymethyl)aminomethane; NaDodSO$_4$, sodium dodecyl sulfate; EDTA, disodium ethylenediaminetetraacetate.

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