Vol. 91, No. 3, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
December 14, 1979 Pages 871-877

COMPARISON OF BLEOMYCIN A_2 AND TALISOMYCIN A SPECIFIC FRAGMENTATION OF LINEAR DUPLEX DNA

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Received October 16, 1979

Summary: The fragmentation of Hind III digested PM2 DNA by treatment with bleomycin and talisomycin has been compared. As observed by electrophoresis on agarose gels, the ethidium bromide staining band patterns produced following incubation of the Hind III PM2 DNA with the drugs differed for bleomycin and talisomycin. These results show that in this system bleomycin and talisomycin treatment of PM2 DNA resulted in breakage of DNA producing different length DNA fragments and may indicate different site specificities for the two drugs.

Introduction: The bleomycins are a group of glycopeptide antibiotics, isolated from Streptomyces verticillus (1) that have been shown to be effective against a variety of neoplasms (2). The primary target for bleomycin (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 (3,4), single strand breakage (3,5), specific and non specific double strand breakage (6,7), noncovalent intermolecular crosslinks (8) and reduction of DNA melting temperature (9). 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 (10).

Talisomycin (TLM) is a new antitumor antibiotic related structurally to BLM. Structures of the two major components of the antibiotic, TLM A and TLM B have been determined. They contain two new amino acids and a unique sugar, 4-amino-4,6-dideoxy-L-talose that have not been previously found in the BLM complex (11). The drug has exhibited significantly greater antibiotic activity against a variety of bacteria and fungi than did BLM (12). Both TLM A and

Reprint requests to C.M.

The abbreviations used are: BLM: Bleomycin; TLM: Talisomycin; DTT: Dithiothreitol; Eth Br: Ethidium Bromide.

0006-291X/79/230871-07$01.00/0

TLM B showed antitumor activity in experimental animal tumor systems (12,13). The mechanism of action of TLM appears to be similar to that of BLM although TLM is 4 to 5 fold less active than BLM in in vitro DNA breakage systems (14). The purpose of the present study was to compare the site specific fragmentation of PM2 DNA induced by TLM with that produced by BLM (7).

Materials and Methods: Covalently closed circular PM2 DNA was isolated as previously described (14). Only PM2 DNA of greater than 90% covalently linked superhelical form (form I) was used. PM2 DNA was incubated in 50mM NaCl, 10mM $MgCl_2$, 14mM dithiothreitol (DTT), 10mM Tris HCL at pH 7.6 with Hind III restriction endonuclease (Boehringer Mannheim, Biochemica, W. Germany) at a concentration of 2 units per mg DNA for 4 hours at 37°. Aliquots of the Hind III PM2 DNA digestion reaction were then incubated in a solution containing BLM A_2 or TLM A (Bristol Laboratories, Syracuse, N.Y.) at 0.5 to 10μM, in a buffer containing 10mM Tris, 20mM NaCl, 40mM DTT at pH 7.6 for selected periods of time at 37°. The reaction was stopped by the addition of an equal volume of a solution containing 12.5ml ethylene diamine tetra-acetic acid (EDTA) 70% glycerol, 0.05% bromphenol blue at pH 7.5 and the mixture was stored at 0°C. Approximately 4μg of PM2 DNA in the final incubation mixture were electrophoreti cally separated on 1% agarose gels under conditions previously described (14). Slab gels were stained with ethidium bromide (Eth Br) dissolved in electrophoresis buffer for a minimum of 2 hours and were then photographed on an ultraviolet light plate (Ultra-Violet Products, Inc., San Gabriel, Mass.). The negative films of the gels were scanned with an RFT Scanning Densitometer Model 2955 (Transidyne General Corporation, Ann Arbor, Mich.) and the relative positions of the ethidium bromide staining DNA fragments determined. Scan settings are indicated in appropriate figure legends.

Results: When PM2 DNA was digested with Hind III restriction endonuclease, 6 of the 7 linear duplex fragments, which are limit products of the reaction (16), were resolved by agarose gel electrophoresis and visualized by fluorescence after staining with Eth Br (Fig. 1, lanes 1 and 10). The fragments ranged in molecular weight from 3.53×10^6 (fragment A) to 1.80×10^3 (fragment F). When Hind III PM2 DNA fragments were subsequently treated with 2 or 5 μM BLM A_2 for 60 or 120 min. (Fig. 1, lanes 2,4,6,8), a series of discrete DNA fragments with intermediate size was produced. Most of the intermediate size fragments migrated to positions in the agarose gel between Hind III fragments A and B and between fragments B and C. These results and the molecular weights of the intermediate size fragments (table 1) as determined from distance migrated in agarose gel electrophoresis relative to the positions of Hind III fragments of PM2 DNA of known molecular weights (15) are in agreement with those reported previously (7).

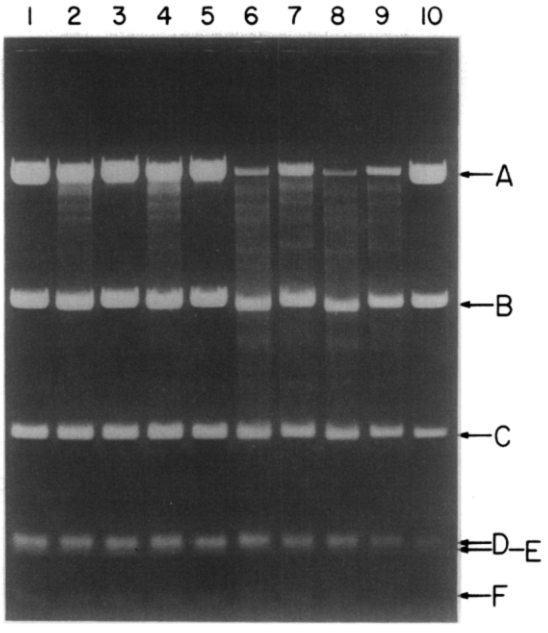

Figure 1. BLM A_2 fragmentation of discrete sites in Hind III digested PM2 DNA. PM2 DNA was digested with Hind III restriction endonuclease followed by BLM A_2 or TLM A at 2 or 5μM for 60 or 120 minutes as indicated in "Materials and Methods". Slots 1 and 10 are control Hind III digested PM2 DNA incubated under drug reaction conditions for 120 min. minus drug in buffer only. Slot 2 is Hind III digested PM2 DNA in the presence of 2μM BLM A_2 for 60 min., slot 3 is 2μM TLM A for 60 min., slot 4 is 2μM BLM A_2 for 120 min., slot 5 is 2μM TLM A for 120 min., slot 6 is 5μM BLM A_2 for 60 min., slot 7 is 5μM TLM A for 60 min., slot 8 is 5μM BLM A_2 for 120 min., slot 9 is 5μM TLM A for 120 min. Hind III fragments of PM2 DNA are indicated in order of decreasing sizes A-F by arrows at the right.

Table 1: Molecular Weights of PM2 DNA Fragments Produced by Hind III Digestion Followed by Bleomycin A_2 or Talisomycin A Treatment

Drug Producing Intermediate Size Fragments	Fragments with Sizes Between Hind III Fragments A and B	Molecular Weight of Intermediate Size Fragments ($X10^{-6}$)
BLM A_2	B-1	3.26±0.03
"	B-2	2.92±0.03
"	B-3	2.65±0.03
"	B-4	2.40±0.03
"	B-5	2.10±0.02
"	B-6	1.84±0.04
TLM A	T-1	3.45±0.03
"	T-2	3.10±0.04
"	T-3	2.80±0.02
"	T-4	2.68±0.04
"	T-5	2.47±0.02
"	T-6	2.20±0.03
"	T-7	1.93±0.02

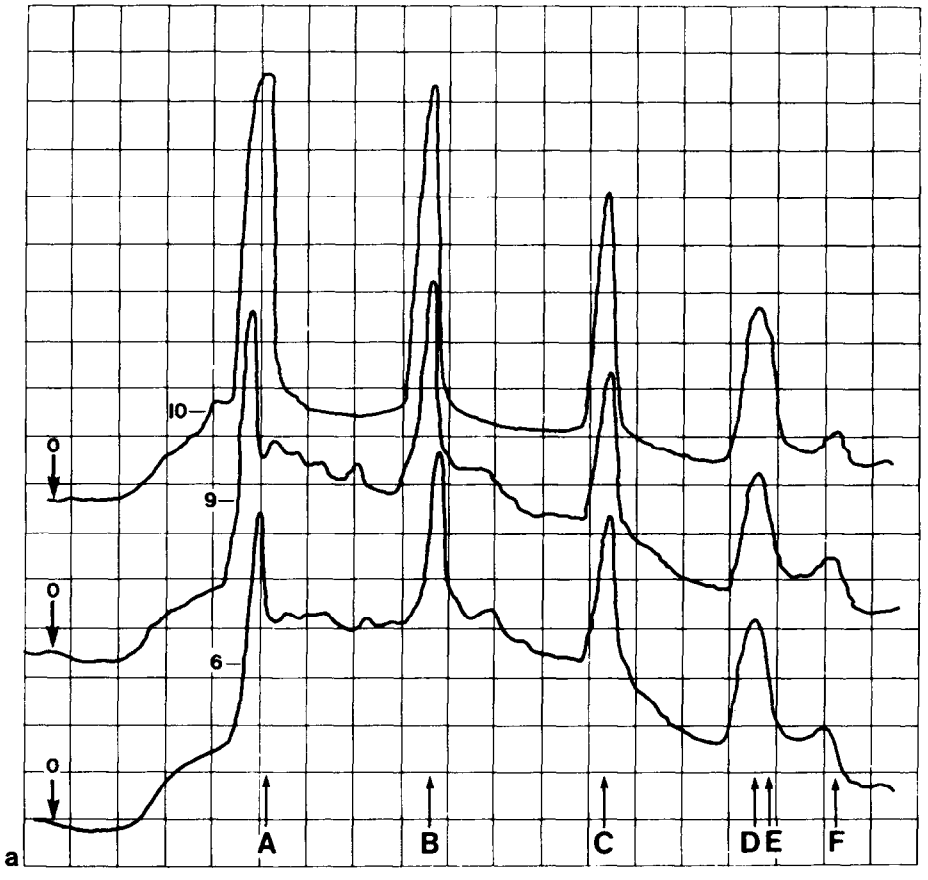

Figure 2. Densitometer scans of Hind III digested PM2 DNA treated with BLM A_2 or TLM A. Figure 2a shows a densitometer scan of slots 7, 9 and 10 of figure 1. Tracing 10 corresponds to slot 10 in figure 1 (Hind III digested PM2 DNA). Tracing 9 corresponds to slot 9 in figure 1 (Hind III digested PM2 DNA treated with TLM A (5μM) for 120 min.). Tracing 6 corresponds to slot 6 in figure 1 (Hind III digested PM2 DNA treated with BLM A_2 (5μM) for 60 min.). Scan settings were: scan speed 0.5CM/sec., gain 7.5, height: 0.1mm, width: 100mm, chart speed 1CM/sec. Figure 2b shows high gain densitometer tracings of the A to B region for Hind III digested PM2 DNA treated with TLM (5μM) for 120 min. (Tracing 9) or BLM (5μM) for 60 min. (Tracing 6). The positions of the Hind III induced fragments are indicated by letters on the abscissa. The bands are identified in Figure 2b and correspond to the fragments identified in table 1.

Hind III PM2 DNA fragments treated with TLM A, under the same conditions used for BLM A_2, also produced a series of discrete DNA fragments of intermediate sizes between fragments A and B (Fig. 1, lanes 3,5,7,9). The intermediate size fragments between Hind III fragments A and B and between fragments B and C following TLM A treatment produced Eth Br staining patterns

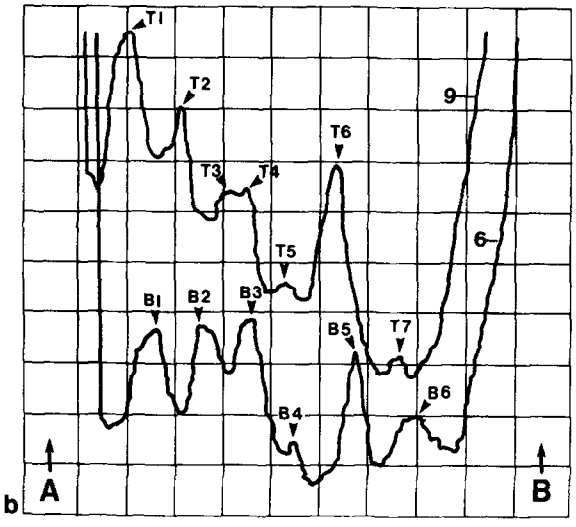

Figure 2 (continued)

in the agarose gel different from those produced by BLM A_2 treatment. The molecular weights of the sub fragments produced by BLM A_2 and TLM A between Hind III and PM2 DNA fragments A and B are compared in table 1.

Figure 2 A shows densitometric scans of agarose gel electrophoresis of products induced by the combined treatment of PM2 DNA by Hind III endonuclease and the drugs. Figure 2 B shows a densitometric scan of the region between Hind III fragments A and B obtained by increasing the sensitivity of the densitometer. The observed differences were reproduced using different batches of PM2 DNA, and in multiple experiments.

These data and the molecular weight determinations shown in table 1 demonstrate that in terms of electrophoretic mobility the TLM A produced sub fragments different from those produced by BLM A_2.

With higher concentrations of BLM A_2 or TLM A and after prolonged incubations, increased Eth Br staining was produced in the gels in the areas between the sub fragments indicating non-specific double strand breakage reactions. However, under these conditions which resulted in increased background fluorescence due to non-specific breakage, the relative positions and patterns of the BLM A_2 and TLM A induced specific cleavage products

were not changed (results not shown). Thus the differences observed could not have been due to partial digestions.

Differences in BLM A_2 and TLM A fragmentation of Hind III digested DNA has also been observed under the same reaction conditions using purified DNA isolated from the E. coli plasmid pBR-322. Moreover, BLM analogs and TLM analogs produced fragmentation equivalent to that induced by BLM A_2, and TLM A respectively (manuscript in preparation). Thus neither the presence of the β-lysine nor changes in the terminal amine moiety were responsible for the changes in site specificity observed between BLM and TLM.

Discussion: The mechanism of action of TLM appears to be similar to that of BLM in that it produces strand breaks in purified isolated DNA and intracellular DNA similar to BLM (14). BLM has been shown to produce specific size fragmentation of PM2 DNA (7). The small number of uniquely positioned BLM fragmentation sites following Hind III digestion suggests the existence of unique sequences of nucleotides in the PM2 genome specifying either the binding of BLM or breakage of DNA by BLM (7). We have demonstrated that TLM also produces specific size fragmentation of PM2 DNA following digestion with the restriction endonuclease, Hind III. However, the size of the resulting fragments differed from those induced by BLM and as determined by electrophoresis of the resulting fragments on agarose gels. That these differences in fragment size were not due to incomplete fragmentation by either drug was demonstrated by the consistency of the Eth Br staining DNA binding patterns on agarose gels following exhaustive BLM and TLM treatment by both increasing concentrations of drug and reaction time. The difference in the sizes of PM2 DNA fragments produced by BLM A_2 and TLM A may be due to a difference in site specificity of fragmentation. This difference in site specificity may result from the structural differences between the two antitumor antibiotics. The findings reported here, and to be reported in a more detailed report suggest that structural alterations near the bithiazole may result in analogs with

different cleavage specificities. Such analogs may be useful as molecular biologic tools, and may have differential cytotoxic effects.

Acknowledgements: The authors thank Ms. Julie Durantini for typographical assistance and A.W. Prestayko, Ph.D. for review of the manuscript. These studies were supported by a grant from Bristol Laboratories.

References:

1. Umezawa, H., Maeda, K., Takeuchi, T. and Oakami, Y. (1966) J. of Antibiotics 19: 200-209.
2. Crooke, S.T. and Bradner, W.T. (1976) J. of Medicine 7: 333-428.
3. Haidle, C.W. (1971) Mol. Pharmacol. 7: 645-652.
4. Muller, W.E.G., Yamazaki, Z., Breter, H. and Zahn, R.K. (1972) European J. Biochem. 31: 518-525.
5. Nagai, K., Suzuki, H., Tanaka, N. and Umezawa, H. (1969) J. Antibiotics, Tokyo, Ser. A. 22:569-573.
6. Povirk, L.F., Wubker, W., Kohnlein, W. and Hutchinson, F. (1977) Nucleic Acids Res. 4: 3573-3580.
7. Lloyd, S.R., Haidle, C.W., Robberson, D.L. (1978) Biochemistry 17: 1890-1897.
8. Lloyd, S.R., Haidle, C.W. and Robberson, D.L. (1979) Proc. Nat'l. Acad. Sci. U.S.A. 76: 2674-2678.
9. Nagai, K., Yamaki, H., Suzuki, H., Tanaka, N. and Umezawa, H. (1969) Biochim. Biophys. Acta 179:165-171.
10. Clarkson, J.M., Humphrey, R.M. (1976) Cancer Res. 36: 2345-2349.
11. Konishi, M., Saito, K., Numota, K., Tsuno, T., Asama, K., Tsakuira, H., Naito, T. and Kawaguchi, H. (1977) J. Antibiotics Tokyo, Ser. A 30: 789-805.
12. Kawaguchi, H., Tsukuira, H., Tomita, K., Konishi, M., Saito, K., Kobaru, S., Numata, K., Fujisawa, K., Miyaski, T., Hatori, M. and Koshiyama, H. (1977) J. Antibiotics, Tokyo, Ser. A 30:779-788.
13. Bradner, W.T. in S. Carter, H. Umezawa, S.T. Crooke (eds) (1979) The Bleomycins-Current Status and New Developments, pp. 333-343 Academic Press, New York.
14. Strong, J.E. and Crooke, S.T. (1978) Cancer Res. 38:3322-3326.
15. Parker, R.C., Watson, R.M. and Vinograd, J. (1977) Proc. Nat'l Acad. Sci. U.S.A. 74:851-855.
16. Brock, C., Eberle, H., Bickle, T.A., Yuan, R. (1976) J. Mol. Biol. 104: 305-311.