Comparison of the Sequences at Specific Sites on DNA Cleaved by the Antitumor Antibiotics Talisomycin and Bleomycin

Christopher K. Mirabelli, Wanda G. Beattie, Cheng-Hsiung Huang, Archie W. Prestayko, and Stanley T. Crooke

Bristol-Baylor Laboratory, Department of Pharmacology [C. K. M., C-H. H., A. W. P.]; Departments of Cell Biology [W. G. B.] and Pharmacology [S. T. C.]; Baylor College of Medicine, Texas Medical Center, Houston, Texas 77131; Bristol Laboratories, Syracuse, New York 13211; [A. W. P.]; and Smith Kline and French Laboratories, Philadelphia, Pennsylvania 19101 [S. T. C.]

ABSTRACT

We have investigated the site-specific cleavage of DNA by the antitumor antibiotics talisomycin and bleomycin by using 5'- or 3'-terminal 32P-labeled restriction fragments of pBR 322 DNA. Both drugs cleaved DNA preferentially at G-C and G-T sequences. However, the relative amounts of cleavage at particular cleavage sites differed between talisomycin and bleomycin at concentrations of the drugs which produced similar extents of total cleavage. In addition, talisomycin produced specific cleavages at G-A sequences which were relatively resistant to cleavage by bleomycin. Within a preferred sequence group (i.e., G-C sequences), some sites were cleaved to a greater extent relative to others by both talisomycin and bleomycin, suggesting that a greater degree of specificity than that provided by only two nucleotides is involved in the site-specific recognition and cleavage of DNA by these drugs.

INTRODUCTION

The BLMs are a group of glycopeptide antibiotics, isolated from Streptomyces verticillus (29) that have been shown to be effective against a variety of neoplasms (3). The primary target for BLM cytotoxicity (3) appears to be interaction with cellular DNA. The effects of BLM on isolated DNA have been shown to include liberation of free bases (5, 20), site-specific and non-specific single- and double-strand breakage (4-6, 10, 11, 14, 19, 22, 28), nonintervalent intermolecular cross-links (12), and a reduction of DNA melting temperature (21). BLM produced breakage of DNA in cells grown in tissue culture, and the extent of degradation was correlated with cell cycle-specific cytotoxicity (23). The primary target for BLM cytotoxicity (3) appears to be interaction with cellular DNA polymerase I (large fragment) (24). The extent of degradation was correlated with cell cycle-specific cytotoxicity of the drug (1). Recently, it has been reported that BLM can preferentially degrade the DNA sequences in the open chromatin within isolated nuclei (9).

TLM are a group of antitumor antibiotics related structurally to the BLMs. The TLMs contain 2 new amino acids and a unique sugar, 4-amino-4, 6-dideoxy-L-talosine, that have not been found previously in the BLM complex (8). TLM A has exhibited significantly greater antibiotic activity against a variety of bacteria and fungi than did BLM A2 (7). The 2 major components of the antibiotic, TLM A and TLM B, have shown antitumor activity in experimental animal tumor systems (7). Like BLM, TLM has been shown to cause DNA breakage in cells grown in tissue culture and single- and double-strand breaks in isolated DNA (13, 17, 25). However, their relative cell cycle specificities (16) and single- and double-strand breakage activities appear to be different (17).

We have shown recently that the site specificities of double-strand fragmentation of DNA by the 2 antitumor antibiotics appear to differ (19). The purpose of the experiments presented here was to determine the nucleotide sequences at the TLM-specific cleavage sites and to compare these with the sites cleaved by BLM.

MATERIALS AND METHODS

Materials. Copper-free BLM A2 and TLM A were obtained from Bristol Laboratories, Syracuse, N.Y.; restriction enzymes Ava I and Msp I were purchased from New England Biolabs, Beverly, Mass.; bacterial alkaline phosphatase was obtained from Bethesda Research Laboratories, Rockville, Md.; polynucleotide kinase was obtained from P-L Biochemicals; DNA polymerase I (large fragment) was obtained from Boehringer Mannheim, Biochemicals, W. Germany; and [γ-32P]ATP (specific activity, ~5000 cpm/mmole) or dCTP (specific activity, 3000 cpm/mmole) were obtained from Amersham/Searle Corp., Arlington Heights, Ill. Covalently closed superhelical plasmid pBR 322 DNA was isolated from Escherichia coli strain Ja 221 according to the procedure of Clewell et al. (2).

Preparation of DNA Restriction Fragments. With the use of standard reaction conditions, the isolated pBR 322 DNA was digested with Ava I restriction enzyme. The 2 terminal ends of the resulting whole linear pBR 322 DNA were labeled with either of 2 methods: (a) the restricted DNA was incubated with bacterial alkaline phosphatase, and the two 5' termini were then phosphorylated with [γ-32P] ATP and polynucleotide kinase; (b) restricted DNA was isotopically labeled at the two 3'-terminal ends with DNA polymerase (large fragment) (24) and [γ-32P]ATP. After labeling the DNA, the DNA was digested with Msp I. The resulting 145- and 61-base-pair fragments (27) each labeled at one 5'-terminal end (Labeling Procedure 1) or at one 3'-terminal end (Labeling Procedure 2) with 32P were isolated and purified from 5% polyacrylamide gels (15).

Fragmentation of DNA by BLM and TLM. The reaction mixture contained 10 mM Tris-HCl (pH 7.5), 10 mM NaCl, 40 mM dithiothreitol, 5'-terminal 32P-labeled restriction fragments (10 ng, 50,000 cpm), 7 μg calf thymus DNA, and either BLM A2 or TLM A at concentrations ranging from 1.0 to 500 μM in a total volume of 10 μl. After incubation for 30 min at 37°, 10 mM EDTA was added to stop the reaction, and the solution was immediately frozen and lyophilized. Each sample was dissolved in 10 μl of a solution of 80% deionized formamide:0.025% bromphenol blue:xylene cyanole. The solution was heated at 90° for 30 sec, and 3-μl aliquots were transferred to 8 or 20% polyacrylamide gels for sequence analysis (15).

Sequence Analysis. The nucleotide sequences of restriction fragments and oligonucleotides produced by BLM A2 and TLM A were determined by the method of Maxam and Gilbert (15). Autoradiography was done by exposing Kodak XRP-5 film to the polyacrylamide gels for 8 to 48 hr at -20°. The sequence of the oligonucleotides was determined by comparison of the chemically degraded restriction fragments,
the nucleotide sequences of which have been reported by Sutcliffe (26). Autoradiographs were scanned with a scanning densitometer (RFT Scanning Densitometer Model 2955; Transidyne General Corporation), and the relative positions and amounts of the ³²P-labeled oligonucleotides were determined.

RESULTS

To compare the specific cleavage sites on DNA produced by BLM and TLM, 2 isotopically labeled DNA fragments were prepared as described in “Materials and Methods.” The pBR 322 restriction fragments used in these experiments were 145 and 61 base pairs long, the sequences of which have been reported by Sutcliffe (26). BLM A₂ and TLM A were incubated with each of the DNA fragments, and the products were analyzed on high-resolution, denaturing, polyacrylamide gels. With the use of the isolated restriction fragments as substrates, the products of base-specific chemical reactions, described by Maxam and Gilbert (15), were separated by gel electrophoresis and used as markers for the products of the drug restriction fragment incubations. By comparing the positions migrated in the gel of the drug produced isotopically labeled subfragments with those of the chemically produced base-specific subfragments, the precise cleavage sites for BLM and TLM were determined.

Using 5 of the specific sequencing reactions described by Maxam and Gilbert (15), G > A, A > G, C + T, C and A > C (Fig. 1 and 2, Lanes 6, 7, and 8). The corresponding nucleotide sequence and the nucleotide length from the 5'-side of bands in autoradiographs which represented cleavages occurred at the 3' side of the nucleotide located directly below the scan peaks.

At higher concentrations of TLM A and BLM A₂, the extent of cleavage of each preferred site relative to one another within the 61-base-pair fragment was equivalent to that observed at lower drug concentrations of both drugs (Chart 1). At relatively high concentrations of both drugs (0.5 to 1.0 μM), the fragment was degraded beyond the extent of “one-hit” kinetics (see “Discussion”). At these high drug concentrations, the intensities of bands in autoradiographs which represented cleavages occurring closer to the labeled 5'-terminal of the fragment increased, relative to those bands produced by cleavage at sites further from the labeled 5'-terminal.

As demonstrated on the 61-base-pair fragment, the preferential cleavage site pattern on the 145-base-pair restriction fragment by BLM A₂ also appears to differ from that of TLM A. This is shown in Fig. 2 within a 51-base-pair portion of the 145-base-pair fragment, which was incubated with increasing concentrations of either BLM A₂ or TLM A, and the resulting subfragments were separated on a 8% polyacrylamide sequencing gel. BLM A₂ cleaved preferentially at G-C and G-T sequences with relatively minor cleavages at other sites such as sequence G-G, positions 32 to 33 (Chart 2, Scan 2). The cleavage pattern produced by TLM A differed from that produced by BLM A₂ (Fig. 2, Lanes 9 and 10). As shown in Chart 2, at a concentration of 200 μM, BLM A₂ produced a relatively greater extent of cleavage than did TLM at G-C sequences 39 to 40, 55 to 56, and 70 to 71, whereas both BLM and TLM produced equivalent amounts of cleavage at G-C sequences 25 to 26, 35 to 36, and 72 to 73. TLM A produced significantly...
greater amounts of cleavage than did BLM A2 at G-T sequences 50 to 51, 62 to 63, and 66 to 67. TLM A also produced cleavage at G-A sequences 27 to 28, 29 to 30, 36 to 37, 41 to 42, and 45 to 46, while BLM A2 produced either no cleavage or relatively little cleavage at the G-A sequences.

The low-level intensities of the bands in Lanes 4 and 12 in Fig. 2 are due to smaller amounts of cpm loaded into these lanes relative to that loaded into Lanes 2, 5, 9, 10, and 12.

The cleavage site specificity of BLM A2 and TLM A and also TLM S2b have also been investigated using the 145-base-pair restriction fragment which was 32P-labeled on the 3' strand of the Aval site (see "Materials and Methods"). The cleavage pattern produced by each of the analogs within a 34-base-pair portion of the fragment is shown in the densitometric scans in Chart 3. The cleavage pattern produced by TLM's A and S2b were equivalent. Both drugs produced extensive cleavage of G-T sequence 78 to 79 and at G-A sequences 52 to 53, 76 to 77, while relatively less cleavage occurred at G-C sequences 55 to 56, 70 to 71, and 72 to 73. The cleavage pattern produced by BLM A2, as represented in the scan shown in Chart 3, is different from the pattern produced by the 2 TLM analogs. G-T sequence 78 to 79 and G-C sequences 55 to 56, and 70 to 71 were cleaved by BLM A2 to extents relatively similar to those observed for the TLMs. However, the G-C sequence 73 to 74 was cleaved more extensively by BLM A2 than the TLMs, while G-A sequences 52 to 53 and 76 to 77 were more susceptible to cleavage by the TLMs. The scans in Chart 3 also contain relatively smaller peaks at other sequences (e.g., A-T, 48 to 49, 74 to 75; G-G, 56 to 57; C-A, 54 to 55) corresponding to sites of relatively low cleavage preference for each of the analogs.

**DISCUSSION**

It has been reported that BLM cleaves both a restriction fragment of bacteriophage φ174 DNA and a segment of the control region of the lactose operon of E. coli DNA, at G-C and G-T nucleotide sequences (4, 28). This is in agreement with our findings using BLM A2 and restriction fragments of pBR 322 DNA. Our findings also demonstrate that the G-C and G-T sequences recognized by BLM A2 differ from one another with respect to the extent to which they are cleaved. However, the extent to which sequences are cleaved relative to one another remained constant over the concentration range of drug used in these experiments (Chart 1).

Like BLM A2, TLM A cleaved the pBR 322 restriction fragments at specific nucleotide sequences, but the preference of specific sequence cleavage differed between the 2 drugs. For example, within the 61-base-pair restriction fragment, one G-T sequence (48 to 49) appeared to be equally susceptible to cleavage at similar concentrations of BLM A2 and TLM A. However, the remainder of the BLM A2-cleaved sequences were relatively resistant to TLM A. It was also observed that within the 145-base-pair restriction fragment, the G-A sequences were cleaved by TLM A but were relatively refractory to cleavage by BLM A2. This relatively greater susceptibility of G-A sequences to cleavage by TLM relative to that by BLM was observed on both strands of the 145-base-pair fragment (Charts 2 and 3). The differences between the cleavage sequence specificities of BLM and TLM do not appear to be a phenomenon of a single particular strand of DNA, because the differences were consistently observed on the 3 different isotopically labeled restriction fragments reported here and also on all other fragments that we have thus far investigated.4

TLM S2b, a TLM analog containing the talo sugar of the TLMs but with a terminal amine moiety equivalent to BLM A2, demonstrated cleavage sequence specificity similar to TLM A (Chart 3). These findings support previously reported data (16, 28).
18) which suggest that the 2 amino acids and the 4-amino-4,6-
dideoxy-L-talose sugar, which are located near the bithiazole
group in TLM but are absent in the BLM structure, are important
in conferring a different site specificity for DNA cleavage by
the TLMs as compared to the BLMs.

As shown in Charts 1 to 3, not all of the preferred cleavage
sites (G-C and G-T for BLM A{sub 2} and G-T, G-A, and G-C for TLM
A) are cleaved to equivalent extents by either of the 2 drugs.
This can be demonstrated by examining the sequence G-A
which was not cleaved in the 61-base-pair fragment (36 to 37)
by TLM A. However, each G-A sequence was cleaved to some
text by TLM A within the 145-base pair fragment. These
differences with respect to the extent of cleavage by either
drug at particular dinucleotide sequences may indicate that a
degree of specificity that than provided by dinucleotide
sequences is involved in the site-specific recognition and cleav-
age of DNA by TLM and BLM. We have mapped the BLM and
TLM sequence-specific cleavage sites on a number of restric-
tion fragments of pBR 322 DNA and are, with the aid of
computer analysis, attempting to gain a clearer understanding
of the role of neighboring sequences at the specific cleavage
sites of these drugs.

The ratio of drug to isotopically labeled DNA used in these
experiments approximated “one-hit” reaction conditions (15)
whereby a majority of the DNA molecules were cleaved at only
one site. This was confirmed from densitometric scans of the
lanes of autoradiographs containing the drug-treated DNA frag-
mants such as those in Figs. 1 and 2. Quantitation of these
scans revealed that within the drug concentration ranges used
in these experiments, approximately one-third (or greater)
of the total radioactivity per lane was located in the bands repres-
resenting the uncleaved 61- or 145-base pair fragments. Under
similar incubation conditions with whole, genomic pBR 322
DNA, the concentrations of drug which were necessary to
produce one break per molecule of DNA, as measured by an
ethidium bromide alkali fluorescence assay, were 45 and 60
nm for BLM A{sub 2} and TLM A, respectively. Therefore, the
approximate 10{sup 5}- to 10{sup 4}-fold increase in drug concentra-
tions needed to produce one-hit reactions on the 61- and 145-base
pair fragments of pBR 322 DNA in comparison with the concen-
trations of drug needed using 4362-base pair whole ge-
nomic pBR 322 DNA may be partially explained by the relative
size differences of the DNA substrate. The relatively small DNA
target size may explain the relatively high concentrations of
BLM used by others (4, 28) investigating the site specificity of
clipage of DNA by TLM and BLM. We have mapped the BLM and
TLM sequence-specific cleavage sites on a number of restric-
tion fragments of pBR 322 DNA and are, with the aid of
computer analysis, attempting to gain a clearer understanding
of the role of neighboring sequences at the specific cleavage
sites of these drugs.

In conclusion, these results demonstrate clearly that the
sequence preference for cleavage of DNA by TLMs A and Sb
differs with that of BLM A{sub 2}. We are currently investigating the
effects of the other structural modifications of this group of
antitumor antibiotics with respect to changes in their site-spe-
cific cleavage of DNA. This information in combination with
other in vitro and in vivo data may aid in the design of new
bleomycin analogs which cleave at highly specific sites in
cellular DNA, resulting in relatively more selective cytotoxic
activities. These analogs may also have potential use as mol-
ecular biological tools in areas such as DNA sequencing.
Furthermore, studies of the structure activity relationships
which determine the site specificities of cleavage for these
agents should provide insights into drug-DNA interactions
within the traditional concept of drug-receptor interactions.

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Fig. 1. Sequence-specific cleavage by TLM and BLM of a 61-base-pair fragment of pBR 322 DNA. The 61-base-pair-long 5'-terminal [$^{32}$P]DNA restriction fragment of pBR 322 was incubated with BLM or TLM (as described in "Materials and Methods"). Equal amounts of DNA and cpm's were loaded alongside standard DNA-sequencing lanes according to the procedures of Maxam and Gilbert (15) on a denaturing 20% polyacrylamide:8 M urea gel. Electrophoresis was carried out for 4 hr at 1500 V. The autoradiograph of the gel is shown. Reactions containing the 61-base-pair fragment include: no drug (Lane 1); BLM A₂, 1 µM (Lane 2); TLM A, 1 µM (Lane 3); BLM A₂, 5 µM (Lane 4); TLM A, 5 µM (Lane 5); base-specific chemical reactions, T + C (Lane 6); C (Lane 7); and A > C (Lane 8); BLM A₂, 25 µM (Lane 9); TLM A, 25 µM (Lane 10); BLM A₂, 50 µM (Lane 11); TLM A, 50 µM (Lane 12); BLM A₂, 100 µM (Lane 13); and TLM A, 100 µM (Lane 14). Solid lines to the corresponding letters indicate the sequence of the fragment as it is read up the gel in the 5' to 3' direction (21).

Fig. 2. Sequence-specific cleavage by TLM and BLM of a 145-base-pair fragment of pBR 322 DNA. The 145-base-pair-long 5'-terminal [$^{32}$P]DNA restriction fragment of pBR 322 was incubated with BLM or TLM and equal amounts of DNA and cpm's (Lanes 4 and 11 contain approximately 25% of total cpm as that in all other lanes.) were loaded alongside DNA-sequencing lanes on a denaturing 8% polyacrylamide:8 M urea gel. Electrophoresis was carried out for 5 hr at 1100 V. The autoradiograph of the gel is shown in this figure. Reactions containing the 145 base pair fragment include: no drug (Lane 1); BLM A₂, 10 µM (Lane 2); TLM A, 10 µM (Lane 3); BLM A₂, 100 µM (Lane 4); TLM A, 100 µM (Lane 5); base-specific chemical reactions, T + C (Lane 6); C (Lane 7); A > C (Lane 8); BLM A₂, 200 µM (Lane 9); TLM A, 200 µM (Lane 10); BLM A₂, 500 µM (Lane 11); and TLM A, 500 µM (Lane 12).
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