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

Christopher K. Mirabelli,[2] Cheng-Hsiung Huang, and Stanley T. Crooke

Bristol-Baylor Laboratory, Department of Pharmacology, Baylor College of Medicine, Texas Medical Center, Houston, Texas 77030 [C. K. M., C. H. H., S. T. C.], and the Bristol Laboratories, Syracuse, New York 13201 [S. T. C.]

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

---

[2] To whom requests for reprints should be addressed.
[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.

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.
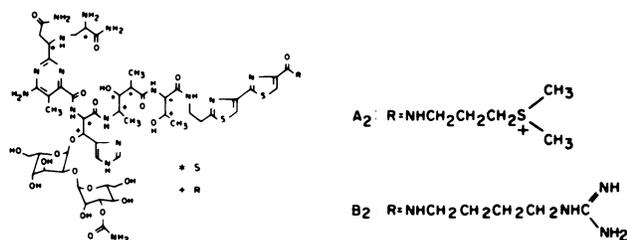
Agarose Gel Electrophoresis. The products of the PM-2

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.

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 3A 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 3B 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.

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.
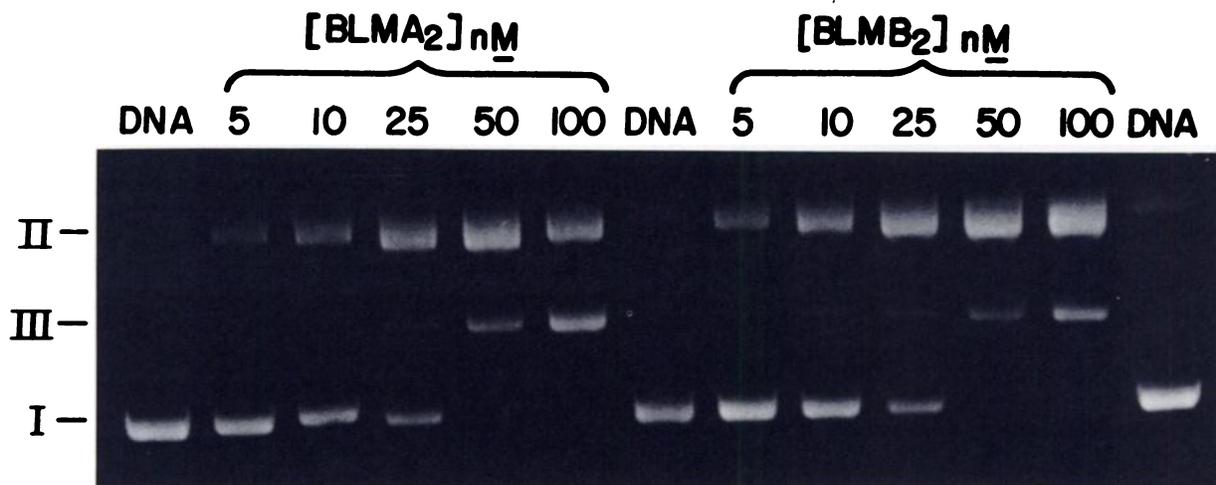[b] Correlation coefficient of best-fit line of probit plot (see text for details).

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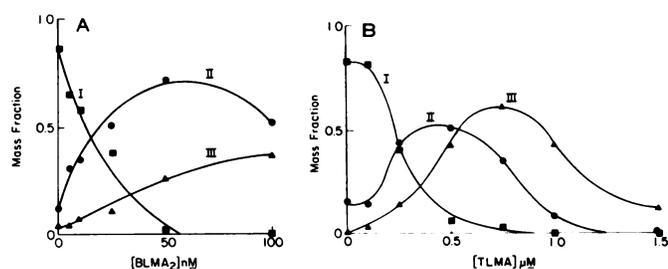
Chart 3. Effect of increasing concentrations of BLM or TLM on PM-2 DNA. Mass fraction of DNA in each form as a function of increasing concentrations of BLM A_2 (A) and TLM A (B).

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

---

[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.

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 PM2 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

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.

ACKNOWLEDGMENTS

REFERENCES

1. Bradner, W. T. Bu-2231, a third generation bleomycin, preclinical studies. *In:* S. Carter, H. Umezawa, and S. T. Crooke (eds.), The Bleomycins—Current Status and New Developments, pp. 333–342. New York: Academic Press, Inc., 1978.
2. Clarkson, J. M., and Humphrey, R. M. The significance of DNA damage in the cell cycle sensitivity of Chinese hamster ovary cells to bleomycin. Cancer Res., *36:* 2345–2349, 1976.
3. Crooke, S. T., and Bradner, W. T. Bleomycin, A review. J. Med. (Westbury), *7:* 333–427, 1976.
4. D'Andrea, A., and Haseltine, W. A. Sequence specific cleavage of DNA by the antitumor antibiotics neocarzinostatin and bleomycin. Proc. Natl. Acad. Sci. U. S. A., *75:* 3608–3612, 1978.
5. Haidle, C. W. Fragmentation of DNA by bleomycin. Mol. Pharmacol., *7:* 645–652, 1971.
6. Haidle, C. W., and Lloyd, S. R. Action of bleomycin on DNA. *In:* S. K. Carter, H. Umezawa, and S. T. Crooke (eds.), The Bleomycins—Current Status and New Developments, pp. 21–34. New York: Academic Press, Inc., 1978.
7. Huang, C. H., Galvan, L., and Crooke, S. T. Interactions of bleomycin analogs with DNA and metal ions by fluorescence quenching. Biochemistry, *19:* 1761–1767, 1980.
8. Iqbal, Z. M., Kohn, K. W., Ewig, R. A. G., and Fornace, A. J. Single-strand scission and repair of DNA in mammalian cells by bleomycin. Cancer Res., *36:* 3834–3838, 1976.
9. Kasai, H., Naganawa, H., Taketa, T., and Umezawa, H. Chemistry of bleomycin. XXII. Interaction of bleomycin with nucleic acids preferential binding to guanine base and electrostatic effect of the terminal amine. J. Antibiot. (Tokyo) Ser. A, *31:* 1316–1320, 1978.
10. Kawaguchi, H., Tsukuria, H., Tomita, K., Konishi, M., Saito, K., Kobaru, S., Numata, K., Fujisawa, K., Miyaski, T., Hatori, M., and Koshiyama, H. Tallysomycin, A new antitumor antibiotic complex related to bleomycin. I. Production, isolation and properties. J. Antibiot. (Tokyo) Ser. A, *30:* 779–788, 1977.
11. Konishi, M., Saito, K., Numata, K., Tsuno, T., Asama, K., Tsukuira, H., Naito, T., and Kawaguchi, H. Tallysomycin, a new antitumor antibiotic complex related to bleomycin. II. Structure determination of tallysomycin A and B. J. Antibiot. (Tokyo) Ser. A, *30:* 789–805, 1977.
12. Litchfield, J. T., and Wilcoxon, F. A simplified method of evaluating dose-effect experiments. J. Pharmacol. Exp. Ther., *96:* 99–113, 1978.
13. Lloyd, S. R., Haidle, C. W., and Robberson, D. L. Bleomycin—specific fragmentation of double-stranded DNA. Biochemistry, *17:* 1890–1896, 1978.
14. Lloyd, S. R., Haidle, C. W., and Robberson, D. L. Site specificity of bleomycin-mediated single-strand scissions and alkali-labile damage in duplex DNA. Gene (Amst.), *7:* 289–302, 1979.
15. Lloyd, S. R., Haidle, C. W., and Robberson, D. L. Noncovalent intermolecular crosslinks are produced by bleomycin reaction with duplex DNA. Proc. Natl. Acad. Sci. U. S. A. *76:* 2674–2678, 1979.
16. Mirabelli, C. K., Mong, S., Huang, C. H., and Crooke, S. T. Comparison of bleomycin A_2 and talisomycin A specific fragmentation of linear duplex DNA. Biochem. Biophys. Res. Commun., *91:* 871–877.
17. Miyaki, M., Morohashi, S., and Ono, T. Single-strand scission and repair of DNA in bleomycin-sensitive and resistant rat ascites hepatoma cells. J. Antibiot. (Tokyo) Ser. A, *26:* 369–373, 1973.
18. Muller, W. E. G., Yamazaki, Z., Breter, H., and Zahn, R. K. Action of bleomycin on DNA and RNA. Eur. J. Biochem., *31:* 518–525, 1972.
19. Nagai, K., Suzuki, H., Tonaka, N., and Umezawa, H. Decrease of melting temperature and single-strand scission of DNA by bleomycin in the presence of 2-mercaptoethanol. J. Antibiot. (Tokyo) Ser. A, *22:* 569–573, 1969.
20. Paika, K. D., and Krishan, A. Bleomycin-induced chromosomal aberrations in cultured mammalian cells. Cancer Res., *33:* 961–965, 1973.
21. Paoletti, C., Lepecq, J. B., and Lehman, I. R. The use of ethidium bromide-circular DNA complexes for the fluorimetric analysis of breakage and joining of DNA. J. Mol. Biol., *55:* 75–100, 1971.
22. Povirk, L. F., Wübker, W., Kohnlein, W., and Hutchinson, F. DNA double-strand breaks and alkali-labile bonds produced by bleomycin. Nucleic Acids Res., *4:* 3573–3580, 1977.
23. Salditt, M., Braunstein, S. N., Camerini-Otero, R. D., and Franklin, R. M. Structure and synthesis of a lipid containing bacteriophage X. Improved techniques for the purification of bacteriophage PM-2 Virology, *48:* 259–262, 1972.
24. Strong, J. E., and Crooke, S. T. DNA breakage by tallysomycin. Cancer Res., *38:* 3322–3326, 1978.
25. Strong, J. E., and Hewitt, R. R. Investigation of human plasma DNase by DNA-polyacrylamide gel electrophoresis. *In:* C. Market (ed.), Isozymes II, pp. 473–483. New York: Academic Press, Inc., 1975.
26. Takeshita, M., Grollman, A. P., Ohtsubo, E., and Ohtsubo, H. Interaction of bleomycin with DNA. Proc. Natl. Acad. Sci. U. S. A., *75:* 5983–5987, 1978.
27. Umezawa, H., Maeda, K., Takeuchi, T., and Oakomi, Y. New antibiotics, bleomycin A and B. J. Antibiot. (Tokyo) Ser. A, *19:* 200–209, 1966.

---

[5] C. K. Mirabelli, C. H. Huang, and S. T. Crooke. Different site specificities for fragmentation of linear duplex DNA by bleomycin and talisomycin analogs, submitted for publication.

[6] C. K. Mirabelli and S. T. Crooke. A comparison of the response of synchronized HeLa cells to talisomycin and bleomycin, submitted for publication.