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Interaction of Bleomycin A_2 with Deoxyribonucleic Acid: DNA Unwinding and Inhibition of Bleomycin-Induced DNA Breakage by Cationic Thiazole Amides Related to Bleomycin A_2†

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ABSTRACT: The association of the antitumor antibiotic bleomycin A_2 with DNA has been investigated by employing several 2-substituted thiazole-4-carboxamides, structurally related to the cationic terminus of the drug. With a 5′-^{32}P-labeled DNA restriction fragment from plasmid pBR322 as substrate, these compounds have been shown to inhibit bleomycin-induced DNA breakage. Analogues possessing 2′-aromatic substituents on the bithiazole ring were more potent inhibitors than those carrying 2′-aliphatic groups, e.g., the acetyl dipeptide A_2. The degree of inhibition was similar at all scission sites on DNA, and inclusion of the analogues did not induce bleomycin cleavage at new sites. DNA binding of bithiazole derivatives has also been studied by two complementary topological methods. Two-dimensional gel electrophoresis using a population of DNA topoisomers and DNA relaxation experiments involving calf thymus DNA topoisomerase I and pBR322 DNA reveal that bleomycin bithiazole analogues unwind closed circular duplex DNA. The inhibition and unwinding studies together support recent NMR studies suggesting that both bleomycin A_2 and synthetic bithiazole derivatives bind to DNA by an intercalative mechanism. The results are discussed in relation to the DNA breakage properties of bleomycin A_2.

The bleomycins are a group of glycopeptide antitumor antibiotics that are used clinically in the treatment of certain cancers [for reviews, see Hecht (1979) and Povirk (1983)]. Their antitumor activity is thought to arise from their ability to degrade DNA. Bleomycins bind and are activated by a variety of transition metal ions including Fe(II). Studies in vitro have demonstrated that the bleomycin–Fe(II) complex forms a redox-active species with O_2 that mediates DNA degradation (Ishida & Takahashi, 1975; Horwitz et al., 1979; Burger et al., 1981).

There is considerable evidence that the bleomycin–Fe(II) complex interacts in a specific manner with double-stranded DNA causing the release of DNA bases (Haidle et al., 1972; Muller et al., 1972; Ishida & Takahashi, 1975; Povirk et al., 1978; Sausville et al., 1978) and the introduction of single- and double-stranded DNA breaks (Suzuki et al., 1968; Nagai et al., 1969; Shirakawa et al., 1971). Thus, although all four bases are released from DNA, bleomycin preferentially liberates thymine > cytosine > adenosine > guanine (Povirk et al., 1978; Sausville et al., 1978). Secondly, DNA strand scission occurs at a limited number of sites with preferential cleavage at GC and GT sequences (D'Andrea & Haseltine, 1978; Takeshita et al., 1978, 1981; Kross et al., 1982a; Mirabelli et al., 1982). The molecular basis underlying DNA specificity exhibited by bleomycins remains to be elucidated.

Several groups have shown that the bleomycin molecule is composed of two functionally distinct domains, shown in Figure 1 for bleomycin A_2, the most common congener (Chien et al., 1977; Kasai et al., 1978; Takita et al., 1978; Glickson et al.,

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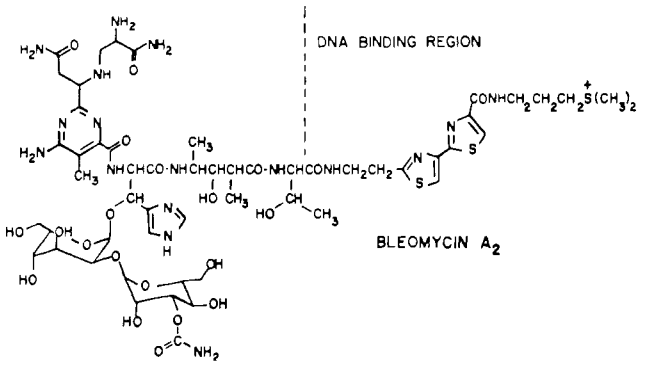

FIGURE 1: Structure of bleomycin A_2.

1981; Sakai et al., 1981). One segment comprising the pyrimidine moiety and associated residues is responsible for chelating the required metal cofactor. In contrast, NMR and fluorescence studies suggest that the region containing the bithiazole moiety is important for binding to DNA. Thus, a C-terminal fragment of bleomycin binds to DNA with a similar affinity to that of the parent drug (Chien et al., 1977; Povirk et al., 1979). Moreover, NMR[1] studies demonstrate the acetyl derivative of the terminal dipeptide fragment [acetyl dipeptide (**1**)] contains all the hydrogen atoms of bleomycin A_2 that undergo perturbation on binding of the antibiotic to the polymer, poly[(deoxyadenylyl)thymidylic acid] [poly(dA-dT), Sakai et al., 1981]. Again, more recent NMR work suggests that the binding of synthetic bleomycin bithiazole derivatives and of the parent drug itself to poly(dA-dT) involves the bithiazole group in an intercalative interaction with duplex DNA and immobilization of the cationic dimethylsulfonium group (Sakai et al., 1981, 1982; Riordan & Sakai, 1983; Booth et al., 1983).

We have sought more detailed information about the binding of bleomycin A_2 to DNA. A number of synthetic dipeptide derivatives of bleomycin A_2 (Figure 2) have been prepared and used as potential inhibitors of bleomycin A_2 induced scission of an end-labeled DNA restriction fragment. The results support the view that the cationic bithiazole C terminus of bleomycin A_2 plays a critical role in DNA binding. In parallel experiments, the nature of the bithiazole–DNA interaction has been characterized by two different topological approaches involving closed circular DNA, one method utilizing a novel two-dimensional gel electrophoresis technique. These studies using DNA substrates of heterogeneous DNA sequence complement previous NMR work employing the synthetic DNA polymer, poly(dA-dT) (Sakai et al., 1981, 1982; Riordan & Sakai, 1983; Booth et al., 1983). The relevance of our findings to the mechanism of bleomycin A_2 induced DNA breakage is discussed.

Materials and Methods

Materials. Plasmid pBR322 DNA was isolated from *Escherichia coli* strain MG1182 by Triton lysis and was purified by banding twice in CsCl–ethidium bromide gradients as previously described (Sakakibara & Tomizawa, 1974). Ethidium bromide was removed by several 1-butanol extractions followed by exhaustive dialysis. The synthesis of cationic thiazole derivatives used in this study has been reported elsewhere (Riordan & Sakai, 1981; Sakai et al., 1981; Riordan & Sakai, 1983). Hygroscopic derivatives were lyophilized

[1] Abbreviations: bp, base pair(s); $CHCl_3$, chloroform; CsCl, cesium chloride; EDTA, ethylenediaminetetraacetic acid; EtdBr, ethidium bromide; NMR, nuclear magnetic resonance; SDS, sodium dodecyl sulfate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

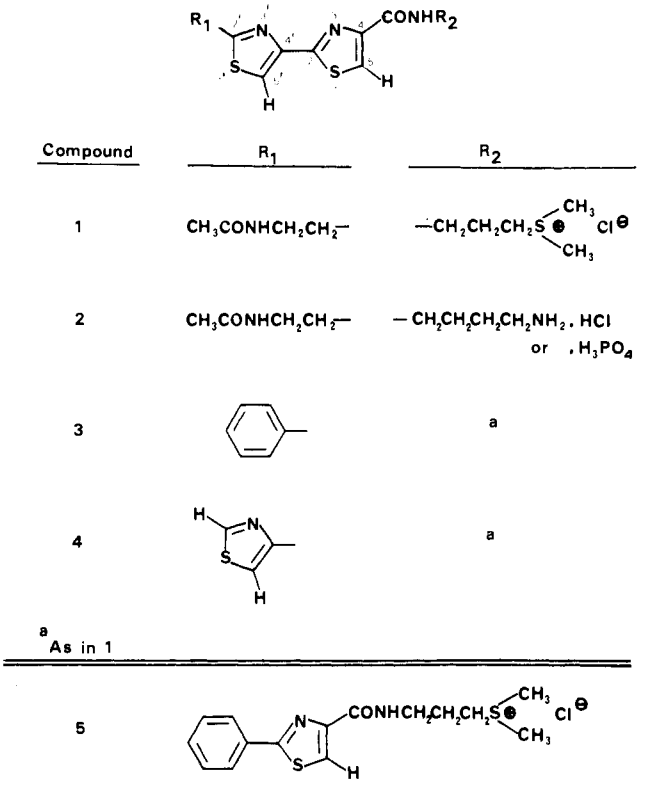

FIGURE 2: Structures of cationic thiazoles used in this work.

overnight prior to use. Low gelling temperature agarose (type VII) was from Sigma.

Bleomycin A_2 was purified from commercial blenoxane (Bristol Laboratories, Syracuse, NY) as previously described (Chen et al., 1980). DNA topoisomerase I from calf thymus nuclei was partially purified by column chromatography on hydroxylapatite followed by phosphocellulose (L. M. Fisher, unpublished results). The preparation was free from nuclease activity and was stored at −70 °C. Restriction enzymes were obtained from New England Biolabs and were used according to the manufacturers instructions. Alkaline phosphatase from calf intestinal mucosa and T4 polynucleotide kinase were from Boehringer and Pharmacia P-L Biochemicals, respectively. [γ-^{32}P]ATP (3000 Ci mmol^{-1}) was obtained from New England Nuclear.

Bleomycin A_2/Fe(II) Induced Breakage of DNA. The 375 base pair *Eco*RI–*Bam*HI restriction fragment from pBR322 labeled at the 5′ *Eco*RI end was obtained as follows. pBR322 (20 μg) was linearized with *Eco*RI restriction enzyme and then treated with alkaline phosphatase for 30 min at 37 °C. Following phenol extraction and ethanol precipitation, the DNA was labeled by incubation with T4 polynucleotide kinase (20 units) and [γ-^{32}P]ATP (100 μCi) in 50 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, and 5 mM dithiothreitol (100 μL), 37 °C, for 30 min. The DNA was ethanol precipitated, digested with *Bam*HI, and run on a 2% low melting agarose gel in TBE buffer (89 mM Tris base, 89 mM boric acid, 2.5 mM Na_2EDTA). The 375-bp *Bam*HI–*Eco*RI band was identified by autoradiography, excised, and isolated by phenol extraction. The fragment was purified on a small (10-μL) DEAE-Sepharose column (equilibrated in 50 mM Tris-HCl, pH 7.5), eluted with 2 M potassium acetate and 50 mM Tris-HCl, pH 7.5, and ethanol precipitated.

Bleomycin A_2/Fe(II) mediated cleavage of DNA was carried out in the presence or absence of bleomycin analogues by the following method. The 5′-^{32}P-labeled *Eco*RI–*Bam*HI fragment from pBR322 was reacted with equimolar concen-

trations of bleomycin A_2 and $FeSO_4$ in 50 mM Tris-HCl and 120 mM KCl, pH 7.5, by incubation at 37 °C for 10 min (total volume 100 μL). The 1:1 bleomycin $A_2/FeSO_4$ solution (7.5 μL) was prepared from freshly made solutions of the components and immediately added to the reaction mixture that had been preequilibrated at 37 °C for 10 min. Reaction was quenched by adding stop solution (20 μL) containing 120 mM Na_3EDTA, pH 8.0, 2 M sodium acetate, and 100 μg mL^{-1} sonicated calf thymus DNA. The DNA was precipitated with ethanol, washed with 95% EtOH, and dried under vacuum.

Samples for gel electrophoresis were dissolved in dye mix containing 50 mM NaOH, 7 M urea, and xylene cyanol, heated to 90 °C for 45 s before loading and electrophoresis in a 10% polyacrylamide/7 M urea denaturing gel (40 cm × 30 cm × 0.5 mm). G+A and C+T chemical sequencing reactions were carried out according to the method of Maxam & Gilbert (1977). Autoradiography was at −70 °C with preflashed Fuji RX X-ray film and a Hannimex light intensifying screen. The degree of inhibition of DNA breakage by inhibitors was determined from the fraction of DNA molecules of full-length size remaining in the treated DNA samples compared to that of the untreated control sample.

Preparation of Topological Isomers of pBR322: Two-Dimensional Agarose Gel Electrophoresis. Plasmid pBR322 topoisomer populations of progressively more negative superhelix density were produced by incubating pBR322 DNA with calf thymus topoisomerase I in the presence of various concentrations of ethidium bromide (0–10^{-6} M) (reaction conditions were as described below for unwinding experiments). For two-dimensional agarose gel electrophoresis, sets of topoisomers of pBR322 with different ranges of linking difference were mixed to yield a population with a broader range of topoisomers. DNA was loaded into a 4 mm × 1.5 mm slot in a 1% agarose gel (20 cm × 20 cm) and electrophoresed in TBE buffer at 60 V for 14 h at 26 °C. The gel was soaked with intermittant agitation over 6–8 h in several changes of TBE buffer containing the bleomycin analogue. Electrophoresis in the second dimension in TBE buffer containing the analogue was carried out as above with circulation of the buffer solution. Experiments involving EtdBr were done in the dark. Gels were stained over several hours in TBE containing EtdBr (1 μg/mL), destained, and photographed under UV light.

DNA Unwinding Experiments. Supercoiled pBR322 DNA (2 μg) was incubated with a calf thymus DNA topoisomerase I preparation (20 units) at 37 °C for 30 min in relaxation buffer (100 μL) containing 50 mM Tris-HCl, pH 7.5, 120 mM KCl, 5 mM dithiothreitol, 0.5 mM Na_3EDTA, 30 μg/mL bovine plasma albumin, and various concentrations of the bleomycin analogue under study. (Prior to addition of the analogue, relaxation solutions were preincubated at 37 °C for 30 min, and a DNA sample was withdrawn to demonstrate full relaxation of plasmid DNA by the topoisomerase.) Reactions were quenched by the addition of 20% SDS (5 μL), and then the samples were extracted twice with an equal volume of neutralized phenol and twice with an equal volume of chloroform. DNA samples (20 μL) were added to electrophoresis dye mix (5 μL) and electrophoresed (3 V/cm) in a 0.8% agarose gel at room temperature or at 1 °C. The electrophoresis buffer contained 40 mM Tris-HCl, pH 8.0, 5 mM magnesium acetate, and 1 mM Na_3EDTA and was circulated while the gel was running. Gels were stained in several changes of TBE buffer containing EtdBr (1 μg/mL) over 3 h, destained in TBE buffer, and photographed.

To show that the extraction procedure was sufficient to remove bound bithiazole derivatives from DNA following relaxation, plasmid pBR322 previously relaxed with topoisomerase I was incubated with the highest levels of EtdBr or bithiazole analogues used in Figure 6. After phenol and $CHCl_3$ extraction and agarose gel electrophoresis, all DNA samples ran identically with untreated relaxed DNA (data not shown).

Table I: Inhibitory Effects of Bithiazole Derivatives on Bleomycin-Mediated Breakage of a 375 Base Pair 5′-End-Labeled DNA fragment

derivative	100% inhibition	50% inhibition	partial
1	5×10^{-2}	5×10^{-3}	
2	5×10^{-3}		
3		10^{-4}	4.3×10^{-5}
4	10^{-3}	10^{-4}	

RESULTS

Inhibition of Bleomycin–Fe(II)-Mediated DNA Breakage by Bithiazole Derivatives. The ability of analogues of the bleomycin bithiazole moiety to act as potential inhibitors of bleomycin-induced DNA breakage was studied with the 375-bp *Eco*RI–*Bam*HI restriction fragment of plasmid pBR322, specifically ^{32}P-labeled at the 5′ *Eco*RI terminus. The use of end-labeled restriction fragments has the advantage that cleavage at many discrete sites can be monitored. To determine conditions for bleomycin A_2–Fe(II)-promoted cleavage, the end-labeled fragment was incubated with equimolar concentrations of antibiotic and Fe(II) in the range 10^{-8} to 5×10^{-6} M. The products of the reactions were separated and displayed by denaturing gel electrophoresis–autoradiography (Figure 3). It is apparent that concentrations of bleomycin A_2–Fe(II) > 5×10^{-8} M result in considerable breakage of the labeled DNA strand. There are several preferred sites of attack, findings that parallel previous studies (D'Andrea & Haseltine, 1978; Takeshita et al., 1978; Kross et al., 1982a; Mirabelli et al., 1982). For inhibition experiments we chose a bleomycin A_2–Fe(II) concentration of 7.5×10^{-8} M, which produces only partial breakage of the DNA restriction fragment.

The effects of compounds **1**–**4** on bleomycin A_2–Fe(II) breakage of the DNA fragment are compared in Figure 4. It is clear that all bithiazole compounds tested inhibited the DNA cleavage reaction although they varied markedly in the concentration required (data presented in Table I). The bleomycin acetyl dipeptide A_2 (**1**) is identical in structure with the DNA binding portion of bleomycin A_2. It completely inhibits DNA degradation at 5×10^{-2} M (Figure 4, lane b) while at 5×10^{-3} M about 50% inhibition was seen (lane c). No inhibition was observed with **1** at 5×10^{-4} M (lane d). Similar results were obtained with the closely related bithiazole derivative **2** (lanes e–g), although at 5×10^{-3} M inhibition by **2** was somewhat greater than that for **1** (compare lanes f and c). Compounds **3** and **4** are structurally similar to **1**, except they carry aromatic rather than aliphatic substituents at the 2′ bithiazole position (Figure 2). Both **3** and **4** were much more effective inhibitors than either **1** or **2**, displaying approximately 50% inhibition at 10^{-4} M concentration (compare lanes i and m with lane c). Inhibition of DNA cleavage by all four bithiazole analogues occurred equally at all cleavage sites and did not change the site specificity of bleomycin A_2 (Figure 4).

Unwinding of Closed Circular Duplex DNA by Bithiazole Derivatives Revealed by Two-Dimensional Gel Electrophoresis. As a first step in examining the interaction of bleomycin bithiazole derivatives with DNA, we studied their effect on the electrophoretic mobilities of a population of topoisomers

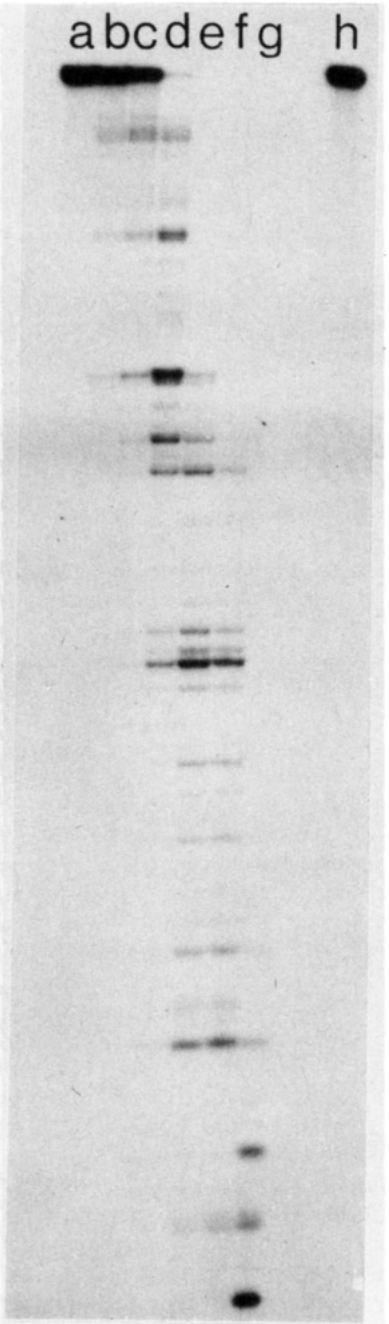

FIGURE 3: Bleomycin A_2 induced degradation of DNA. The 375-bp 5′-^{32}P end-labeled DNA fragment (4000 cpm) was incubated with equimolar Fe(II)–bleomycin A_2 for 10 min at 37 °C. Reactions were quenched by the addition of 10 mM EDTA, and the DNA was displayed on a denaturing 10% polyacrylamide–urea gel. Concentrations of 10^{-8}, 2.5×10^{-8}, 5×10^{-8}, 10^{-7}, 2.5×10^{-7}, 5×10^{-7}, and 5×10^{-6} M Fe(II)–bleomycin A_2 were used for lanes a–g, respectively. Lane h contained untreated 375-bp 5′-^{32}P end-labeled DNA, processed similarly.

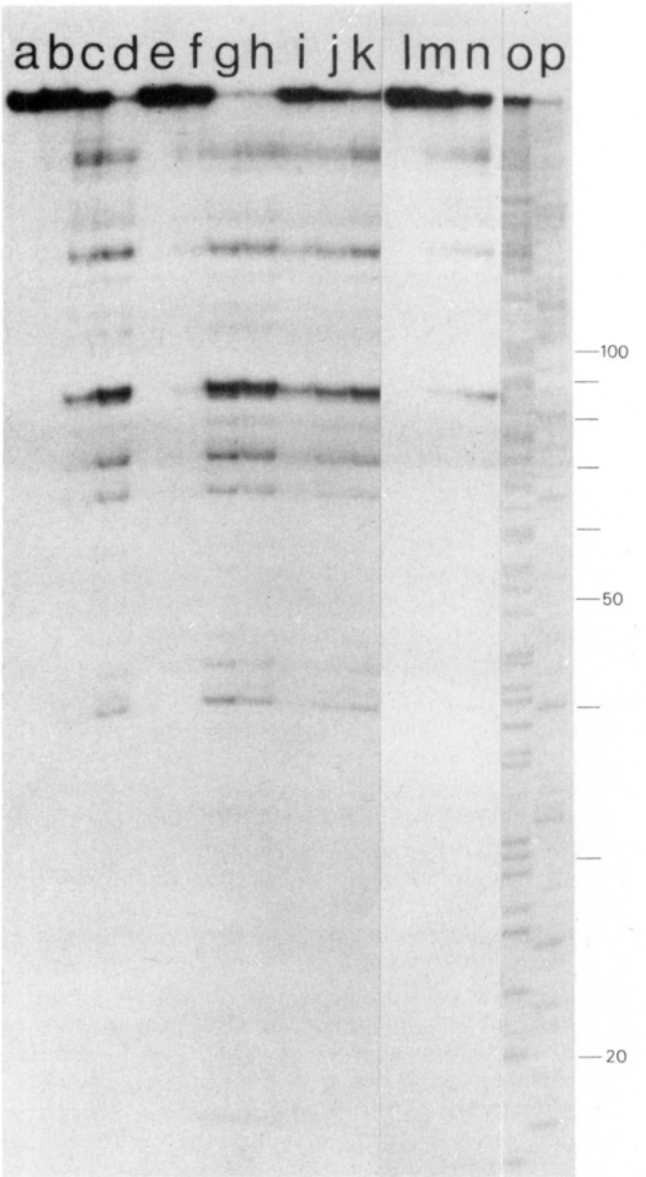

FIGURE 4: Inhibition of bleomycin-promoted DNA degradation. The 375-bp 5′-^{32}P end-labeled restriction fragment was incubated with bithiazole analogues at 37 °C for 10 min before addition of 7.5×10^{-8} M bleomycin A_2–Fe(II) and incubation for a further 10 min. The DNA was analyzed on a denaturing 10% polyacrylamide gel. The restriction fragment was incubated in the absence (a) or presence (b–n) of bleomycin. Lanes b–d contained compound **1** at 5×10^{-2}, 5×10^{-3}, and 5×10^{-4} M; lanes e–g contained **2** at 5×10^{-2}, 5×10^{-3}, and 5×10^{-4} M; lanes i–k contained **3** at 10^{-3}, 10^{-4}, and 10^{-5} M; lanes l–m contained **4** at 10^{-3}, 10^{-4}, and 10^{-5} M, respectively. Lane h had no bithiazole inhibitor added. Lanes o and p are the G+A and C+T Maxam–Gilbert sequencing reactions. Numbers denote nucleotide positions in the DNA sequence of pBR322 (Sutcliffe, 1979). Bleomycin A_2 induced breakage occurs to the 3′ side of nucleotide positions 16, 20, 31, 39, 41, 42, 45, 49, 55, 63, 64, 69, 70, 77, 78, 82, and 84, i.e., predominantly between 5′-GC and 5′-GT.

of pBR322. Helix unwinding caused by drug binding alters the writhing of a closed circular DNA, which can be most conveniently detected by two-dimensional gel electrophoresis. A mixture of topoisomers of pBR322 spanning a broad range of linking numbers was electrophoresed along one edge of a slab agarose gel. After the gel was soaked in electrophoresis buffer containing the DNA ligand, electrophoresis was carried out in the second orthogonal direction at the same, constant temperature.

The results of such an experiment are illustrated for the known intercalator ethidium bromide in Figure 5. Inclusion of EtdBr in the second dimension displaces individual topoisomers off the diagonal and resolves them forming adjacent spots that lie on a smooth curve (Figure 5B). This pattern is observed because in the second dimension positively supercoiled DNA has a higher mobility due to the increase in superhelical density caused by EtdBr binding, whereas the migration of negatively supercoiled DNA is retarded as this DNA is relaxed by intercalation of EtdBr. In the first dimension topoisomer **5** (Figure 5B) corresponds to the position of relaxed DNA. Under these electrophoresis conditions, topoisomers **1**–**4** were positively supercoiled whereas topoisomers > **6** were negatively supercoiled. In the second di-

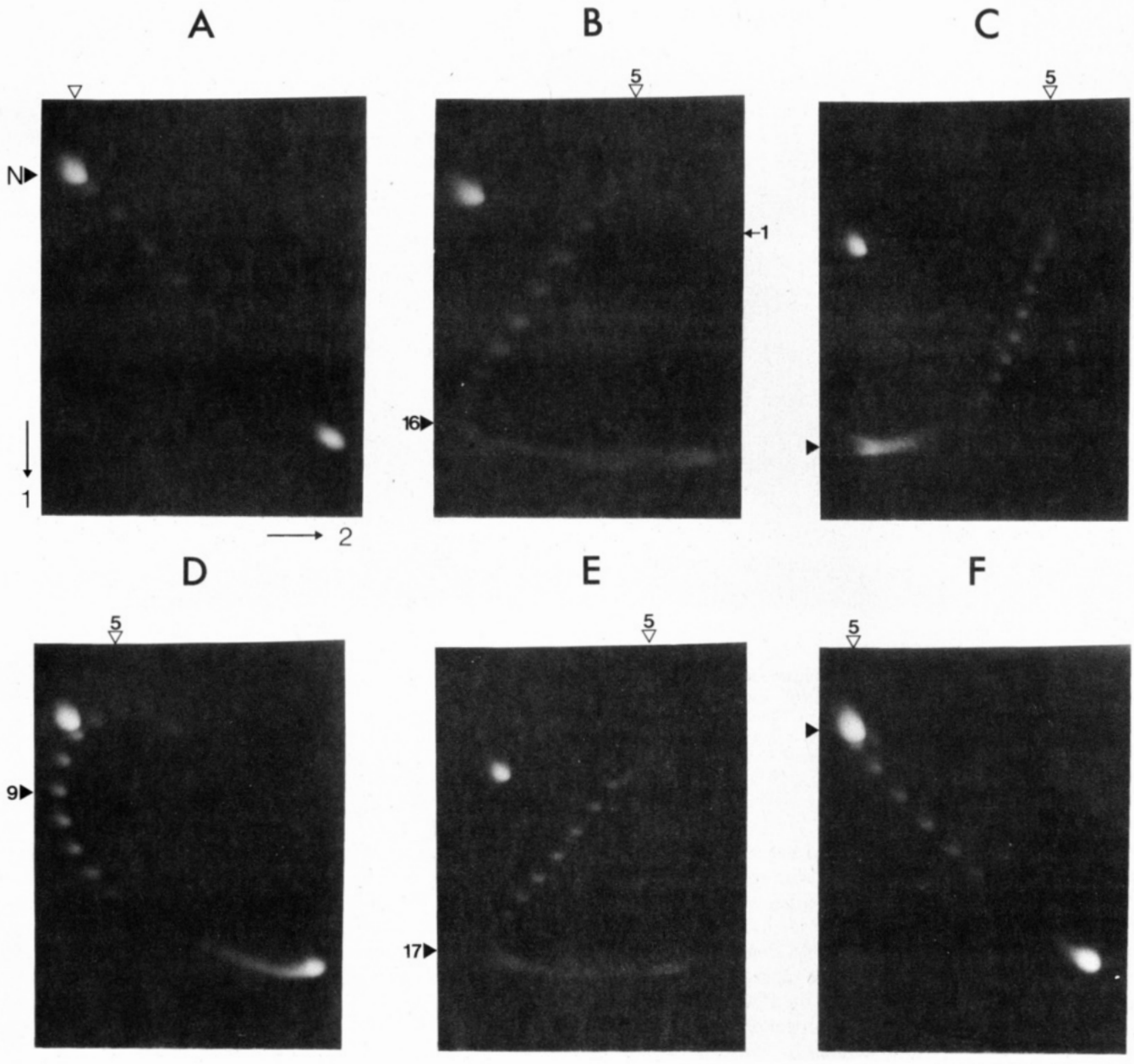

FIGURE 5: Two-dimensional gel electrophoresis. A mixture of negatively and positively supercoiled topoisomers of pBR322 (see Materials and Methods) was electrophoresed in the first dimension under standard conditions. Gels were soaked in appropriate concentrations of EtdBr or bithiazole derivatives contained in the same electrophoresis buffer and reelectrophoresed in the second dimension. (A) No addition in the second dimension; (B and C) EtdBr added at 1.5×10^{-8} and 4×10^{-8} M; (D and E) **3** included at 5×10^{-6} and 1.5×10^{-5} M; (F) **2** included at 1.5×10^{-5} M. Open and filled triangles depict the position of relaxed DNA in the first and second dimensions, respectively. N denotes nicked DNA. Topoisomers are labeled in the order of decreasing linking number.

mension, DNA unwinding by EtdBr results in topoisomer **16** running at the relaxed position, topoisomers **1–15** being positively supercoiled and topoisomers > **17** being negatively supertwisted. Higher EtdBr concentrations (Figure 5C) result as expected in more pronounced overwinding of circular DNA. The technique graphically illustrates the concommitant overwinding of closed circular DNA resulting from ligand intercalation into the DNA helix.

Bithiazole derivatives were examined by the same technique, and the results obtained for **3** are shown in Figure 5D,E. Inclusion of **3** produced a two-dimensional separation of DNA topoisomers consistent with DNA helix unwinding and compensating positive supercoiling of DNA caused by binding of **3** to the DNA. With **3** at 5×10^{-6} and 1.5×10^{-5} M, topoisomers **9** and **17**, respectively, ran at the position corresponding to relaxed DNA in the second dimension (Figure 5D,E). Therefore, these bithiazole concentrations unwind DNA by 4 and 12 helical turns, respectively, suggesting a linear dependence of DNA unwinding on ligand concentration under these conditions. Compound **4**, which like **3** carries an aromatic substituent at the 2′-position of the bithiazole system, behaved similarly to **3**, producing a double-arch pattern at 1.5×10^{-5} M like that of Figure 5E with topoisomer **17** running at the relaxed position in the second dimension (not shown). Thus, **3** and **4** induce comparable unwinding of duplex DNA.

Compounds **1** and **2** gave less pronounced effects on the migration of DNA topoisomers than **3** and **4**, as seen for **2** in Figure 5F. Nevertheless, displacement of both positively and negatively supercoiled DNA species from diagonal positions is consistent with DNA helix unwinding by these derivatives.

DNA Unwinding by Bithiazole Derivatives Detected following Relaxation with DNA Topoisomerase I. It is important to examine whether bithiazole derivatives bind and unwind DNA under the same conditions in which they inhibit

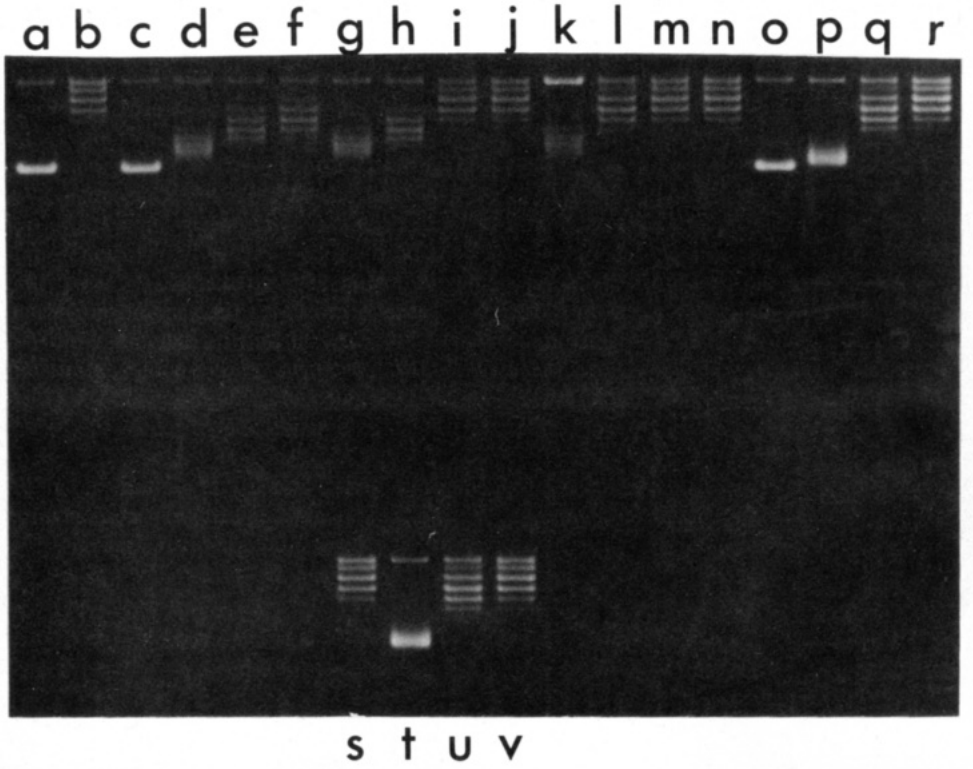

FIGURE 6: Bithiazole derivatives induce supercoiling of closed circular DNA. Negatively supercoiled pBR322 DNA was relaxed by incubation with calf thymus topoisomerase I at 37 °C for 30 min and the reaction either terminated (lane b) or incubated with ethidium bromide or bithiazole analogues for a further 30 min (lanes c–v). The topoisomerase was inactivated and the DNA phenol extracted to remove bound ligand prior to analysis by agarose gel electrophoresis in Tris–acetate–Mg^{2+} buffer at room temperature. Lanes c–f contained EtdBr at 10^{-6}, 10^{-7}, 5×10^{-8}, and 2.5×10^{-8} M; lanes g–i contained **3** at 10^{-4}, 4.3×10^{-5}, and 4.3×10^{-6} M; lanes k–n contained **1** at 5×10^{-3}, 5×10^{-4}, 5×10^{-5}, and 5×10^{-6} M; lanes o–r contained **2** at 5×10^{-2}, 5×10^{-3}, 5×10^{-4}, and 5×10^{-5} M; lanes t–v contained **5** at 10^{-3}, 10^{-4}, and 10^{-5} M, respectively. No additions were made to lanes j and s. Lane a contained untreated supercoiled pBR322 DNA, processed as above.

bleomycin A_2–Fe(II)-promoted DNA degradation. Plasmid pBR322 was relaxed by incubation with calf thymus DNA topoisomerase I prior to addition of bithiazole analogues and further incubation using essentially identical conditions of buffer and temperature as in the DNA degradation experiments. Following inactivation of the topoisomerase and removal of the potential DNA ligand, the DNA was analyzed by agarose gel electrophoresis. DNA topoisomerase I removes positive superhelical turns generated by ligand-induced unwinding of circular DNA. Subsequent removal of ligand results in the formation of negatively supercoiled DNA. From Figure 6, it is apparent that in the presence of topoisomerase I, **1**, **2**, **3**, and **5** all induce supercoiling of pBR322 DNA. To determine the sign of DNA supercoiling, the DNA was electrophoresed at 1 °C (Figure 7). The effect of temperature on the helical pitch of DNA is such that a closed circular DNA is more negatively supertwisted at lower temperature (Wang, 1969; Liu & Wang, 1978). It is clear from comparison of Figures 6 and 7 that all the samples migrate more rapidly relative to the nicked position at the lower temperature, proving that they are negatively supertwisted. In fact the relative increase in mobility is in the same direction as that seen for DNA relaxed in the presence of EtdBr, a well-characterized DNA unwinding agent (lanes c–f). These results confirm the observations made with two-dimensional gel electrophoresis that bleomycin bithiazole analogues unwind DNA.

It is gratifying that there is good agreement between the relative efficiencies of DNA unwinding by DNA ligands determined by two-dimensional gel electrophoresis and by using DNA topoisomerase I. Thus, EtdBr is approximately 10^3 times more effective in promoting DNA unwinding than compound **3** under our conditions (Figures 6d,e,g,h and 5B,E). Moreover, **3** is 50 times more effective than **1** (Figure 6g,k), and **2** at 5×10^{-3} M (lane p) resulted in rather more unwinding than the same concentration of **1**. Interestingly, the phenylthiazole **5** also unwinds DNA (Figure 6t,u). It may be noted that the concentrations of bithiazole analogues used to observe DNA unwinding (Figure 6) are very similar to those required to inhibit bleomycin A_2–Fe(II)-mediated DNA degradation (Figure 4).

DISCUSSION

The DNA binding region of bleomycin A_2 is believed to reside in the cationic terminal dipeptide of the molecule, and association of this segment with DNA is thought to be required for DNA degradation. Consistent with this view, we have shown that close analogues of the cationic terminus act as inhibitors of bleomycin A_2 promoted DNA degradation. The inhibitory potentials for the compounds examined follow the order **3**,**4** $\gg$ **2** > **1** showing that the degree of inhibition depends on the substituents carried by the bithiazole ring system. Derivatives **1** and **2** possess aliphatic substituents at the 2′-position of the bithiazole system and were moderately good inhibitors, even though the acetyl dipeptide **1** is identical with the cationic terminus of bleomycin A_2. These results can be compared with the recent work of Kross et al. (1982b), who also studied **1** and **2** as inhibitors of bleomycin A_2 degradation employing experimental conditions almost identical with those used here. They showed that **2** was somewhat more potent than **1** as an inhibitor of bleomycin-mediated [^{3}H]thymine release from PM2 DNA. Moreover, using an end-labeled restriction fragment they found that **1** at 6×10^{-3} M gave 50% inhibition of DNA breakage by bleomycin A_2, in gratifying agreement with the value of 5×10^{-3} M obtained here.

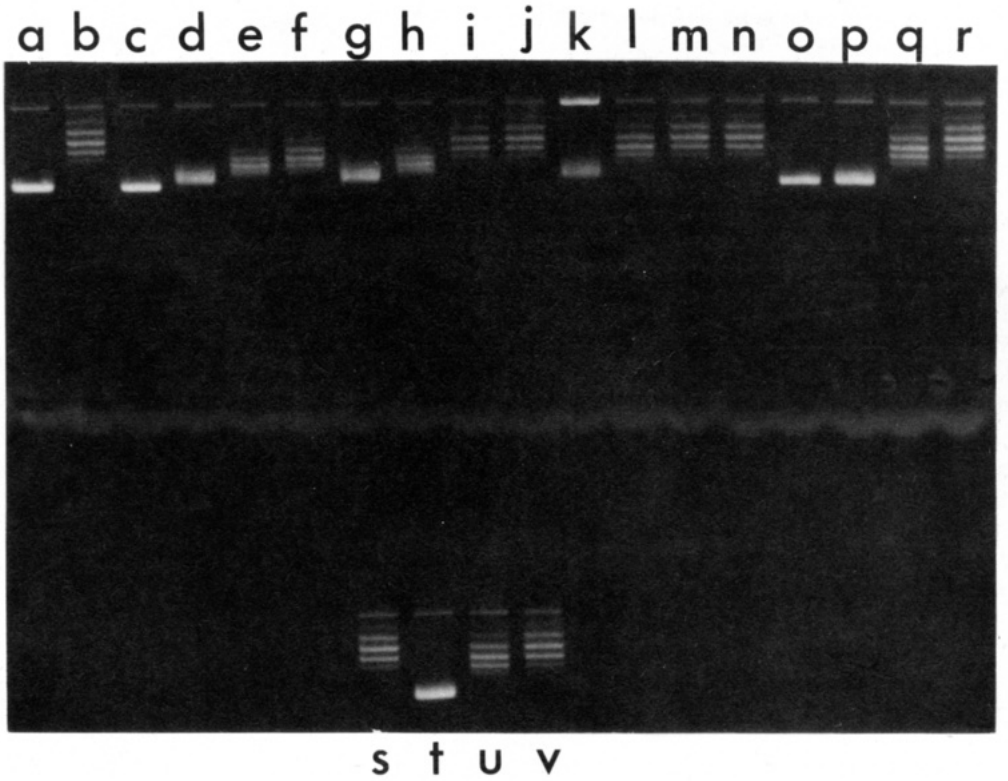

FIGURE 7: Low-temperature gel electrophoresis reveals that bithiazole analogues unwind closed circular DNA. Agarose gel electrophoresis of the DNA samples was carried out as described in the legend of Figure 6 except the temperature was maintained at 1 °C. All the DNA samples migrate closer to the supercoiled rather than nicked position when compared to their mobilities in Figure 6. Thus, all DNA species in Figure 6 are negatively rather than positively supercoiled (see Results).

Interestingly, **3** and **4**, which bear aromatic 2′-substituents, were 50-fold more potent in inhibiting bleomycin A_2 induced DNA breakage than **1**, the acetyl dipeptide A_2. The greater effectiveness of **3** and **4** may be attributed in part to their greater affinity for DNA compared to **1**, as demonstrated by binding measurements (Sakai et al., 1982).

Previous work indicates that the structures of complexes formed between DNA and dipeptide derivatives are slightly different for each bithiazole analogue. Thus, ^{1}H NMR studies have shown that when bleomycin A_2 or cationic dipeptide derivatives bind to poly(dA-dT), the bithiazole proton resonances are broadened and shifted to high field showing that they experience the ring-current effects of stacked nucleotide base pairs. The result has been interpreted as arising from intercalation of the bithiazole moiety into the DNA duplex. However, the magnitudes of the high-field shifts are different for the various bithiazole derivatives, suggesting that the structure of the complex, particularly the degree of intercalation, is somewhat different for each bithiazole analogue (Riordan & Sakai, 1983; Sakai et al., 1982). The NMR data indicate that the presence of 2′ aliphatic substitutents in bleomycin A_2 and the acetyl dipeptide **1** imposes steric constraints on the ability of these molecules to intercalate with duplex DNA. However, bithiazole analogues with an aromatic ring at the 2′-position, i.e., **3** and **4**, exhibit the largest high-field shifts for aromatic protons observed for bithiazole analogues. Since protons on the 2′ aromatic ring substituent also experience ring-current effects, it has been proposed that the three contiguous rings in **3** (C6–C16 distance of 11.56 Å) and **4** intercalate into DNA spanning the entire base-paired region of poly(dA-dT), in contrast to other bithiazole derivatives that are thought only partially to span the DNA duplex (interstrand phosphate–phosphate distance of 15.9 Å) (Sakai et al., 1982; Riordan & Sakai, 1983). Evidence supporting this idea has come from the X-ray crystal structure determination of **3**, which in the crystal adopts a conformation with all three aromatic rings coplanar (Kuroda et al., 1982). Moreover, molecular modeling using energy-minimization techniques shows that the envisaged intercalation of **3** into duplex DNA is energetically feasible (Kuroda et al., 1982). According to this model, and in agreement with NMR data, the phenyl ring of **3** extends into the base-paired region; the smallest interaction with DNA base pairs is exhibited by the first thiazole ring, with the second thiazole ring showing an intermediate extent of intercalation. These considerations may explain the greater DNA affinity of **3** and **4** compared to **1** and the greater inhibitory potential of these analogues.

One consequence of an intercalative mode of binding to DNA by bleomycin A_2 and dipeptide A_2 analogues is that the DNA helix should undergo concomitant unwinding. As dipeptide derivatives do not cause DNA strand scission, topological methods exploiting the properties of closed circular DNA can be used to detect unwinding of DNA induced by binding of these compounds. Using two different topological techniques we find that binding of bithiazole derivatives to pBR322 DNA does indeed induce DNA helical unwinding. With DNA topoisomerase I, under the conditions of the bleomycin A_2 promoted DNA degradation experiments, the derivatives follow the unwinding order $\mathbf{3,4} \gg \mathbf{2} > \mathbf{1}$, the same as that observed for the inhibition potential of the compounds. The experiments suggest that intercalative binding of these analogues to DNA is responsible for inhibition of bleomycin A_2 activity.

DNA unwinding as a criterion for DNA intercalation is strengthened if it can be shown that unwinding of a closed circular DNA in the presence of the DNA ligand induces relaxation and then overwinding (positive supercoiling) of the DNA circle (Waring, 1981). Previously, relaxation and overwinding of circular DNA by intercalative agents has been monitored by rather laborious techniques such as sedimentation

analysis. We have used two-dimensional agarose gel electrophoresis in analyzing the effects of bithiazole derivatives on a population of DNA topoisomers spanning a range of linking difference. One advantage of the method is that relaxation and overwinding of closed circular DNA can be examined on one gel with a single ligand concentration in the second dimension. The method is rapid, simple, and very sensitive. By using this approach, we have confirmed that all the bithiazole derivatives we tested do promote helix unwinding and overwinding of closed circular DNA as required for intercalative binding. These studies demonstrate the utility of two-dimensional gel electrophoresis, a technique until now largely employed to follow conformational changes in circular DNA (Wang et al., 1982; Courey & Wang, 1983).

In previous work on bleomycin A_2, evidence has been presented that binding of the drug to supercoiled ColE1 DNA at pH 5.5 (to suppress the DNA breakage activity of the antibiotic) results in relaxation of negative supercoils (Povirk et al., 1979). Higher concentrations of bleomycin produced recoiling of DNA. This behavior was ascribed to DNA intercalation by the bithiazole moiety. However, these studies were carried out in buffers of very low ionic strength. It is likely that the binding of bleomycin and bithiazole derivatives is very sensitive to nucleic acid structure under these conditions. Our experiments were conducted at much higher salt and buffer concentrations (50 mM Tris, 120 mM KCl) more closely approximating the in vivo situation.

The importance of the bithiazole moiety in DNA binding by bleomycin A_2 is supported by this work and several previous studies. At least two aromatic rings appear to be necessary for efficient binding to DNA since no binding to poly(dA-dT) is observed for monothiazole model compounds (Sakai et al., 1982; Riordan & Sakai, 1983). Interestingly, the phenylthiazole derivative **5** binds to DNA causing unwinding (Figure 6) and acts to inhibit bleomycin A_2 mediated DNA breakage (not shown). Thus, in agreement with ^1H NMR studies (Riordan & Sakai, 1983), these results show this molecule containing two different aromatic rings undergoes intercalation into DNA.

An important unresolved question is the mechanism by which bleomycin A_2 expresses its preference for DNA breakage at GC and GT sequences. It is not known whether this sequence preference accrues from a binding preference of the terminal dipeptide moiety. However, it is significant that all the bithiazole derivatives we studied appeared to inhibit uniformly DNA breakage by bleomycin at all its cleavage sites (Figure 4). Moreover, increasing concentrations of inhibitor did not alter the site specificity of breakage by the antibiotic. Similar findings were reported by Kross et al. (1982b). The results are in contrast with the effects of other unrelated intercalators, e.g., ethidium bromide, actinomycin D, and distamycin A (Sugiura & Suzuki, 1982), and of the platinum anticancer drug cis-diamminedichloroplatinum(II) (Mascharak et al., 1983), all of which induce new sites of DNA breakage by bleomycin A_2. These observations can be rationalized if, as seems likely, close analogues of the cationic terminus bind to DNA with the same or similar sequence specificity to bleomycin A_2 itself.

Clearly, more detailed experiments must be carried out to address the question of site-specific binding and DNA cleavage by bleomycin A_2. Understanding of the DNA recognition properties of the drug may then facilitate the rational design of bleomycins of various specificities and enhanced anticancer activity.

Registry No. **1**, 76275-84-8; **2**, 82326-32-7; **3**, 96394-71-7; **4**, 80337-68-4; **5**, 85318-76-9; bleomycin A_2, 11116-31-7.

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DNA Damage and Growth Inhibition in Cultured Human Cells by Bleomycin Congeners†

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ABSTRACT: Bleomycin is hypothesized to cause cell growth inhibition and cell death via DNA cleavage. We have attempted to determine if net DNA cleavage is directly related to growth inhibition by measuring whether both parameters vary in parallel. Of primary importance to these studies was use of several bleomycin congeners. We have shown that these congeners vary in their abilities both to inhibit cell growth and to cause DNA damage. Bleomycin B_2, tallysomycin, and phleomycin were the most potent growth inhibitors, and bleomycin B_2 caused the most DNA damage. *N*-Acetylbleomycin A_2 was inactive in both assays. The net amount of DNA damage measured at two levels of growth inhibition was compared for each congener and was found to vary widely among the congeners. Similarly, the degree of growth inhibition at a given level of submaximal DNA damage was found to vary widely when individual congeners were compared to each other. Hence, growth inhibition and net DNA damage due to bleomycin are not directly correlated with each other when individual congeners are compared to each other.

The bleomycins are glycopeptide-derived antitumor antibiotics isolated from cultures of *Streptomyces verticillus* (Umezawa et al., 1966). Blenoxane, a mixture of bleomycin A_2 (60–65%), bleomycin B_2 (~30%), and several other bleomycins, including demethylbleomycin A_2 (Crooke & Bradner, 1976), is used clinically for the treatment of certain tumors (Carter et al., 1978; Umezawa et al., 1972; Umezawa, 1976). Additional bleomycin group antibiotics investigated as potential antitumor agents include the phleomycins (Bradner & Pindell, 1962; Umezawa et al., 1962) and tallysomycins (Kawaguchi et al., 1977; Konishi et al., 1977). Bleomycins, phleomycins, and tallysomycins are all known to induce breakage of chromosomal DNA (Kross et al., 1982b). Strand scission occurs both in vitro (D'Andrea & Haseltine, 1978; Sugiura & Suzuki, 1982; Suzuki et al., 1969) and in vivo (Iqbal et al., 1976; Suzuki et al., 1969; Terasima et al., 1970), and both single-strand and double-strand breaks are observed (Haidle, 1971; Suzuki et al., 1969; Terasima et al., 1970). Breakage is sequence selective (D'Andrea & Haseltine, 1978), but preferred cleavage sites differ somewhat among the three structural families (Kross et al., 1982b; Mirabelli et al., 1979, 1980, 1982a,b); the precise mechanism of DNA strand scission remains to be established.

Bleomycin-induced DNA scission is hypothesized to cause inhibition of cell growth (Barlogie et al., 1976; Barranco & Humphrey, 1971; Hittelman & Rao, 1974) and to cause cell death (Clarkson & Humphrey, 1976). Cells are accumulated in late G_2 phase of the cell cycle when treated with bleomycin under conditions which also cause DNA breakage (Burger et al., 1982; Hittelman & Rao, 1974). Inhibition in mitosis has also been observed (Tobey, 1972), and recovery from inhibition is very slow. Bleomycin also produces a dose-dependent decrease in colony-forming ability (Terasima et al., 1972). HeLa S_3 cells were less sensitive than L5 cells (Terasima & Umezawa, 1970), and the Cu(II) complex was more active against Ehrlich ascites cells than was metal-free bleomycin; metal-free bleomycin was more active than the Zn(II) or Fe(II) complexes (Rao et al., 1980).

A direct causative relationship between bleomycin-induced DNA damage and growth inhibition or cell death has not been conclusively established. This is true in most cases because carefully controlled experiments have not been performed to measure both parameters simultaneously. We have undertaken to do so in order to establish some basic correlations between bleomycin A_2 (BLM A_2)[1] induced growth inhibition and DNA strand breakage in cultured human cells. Additionally, to obtain information that may bear relevance to structural features of bleomycins that contribute to their antineoplastic

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[1] Abbreviations: demethyl-BLM A_2, demethylbleomycin A_2; BLM A_2, bleomycin A_2; BLM B_2, bleomycin B_2; iso-BLM A_2, isobleomycin A_2; epi-BLM A_2, epibleomycin A_2; *N*-Ac-BLM A_2, *N*-acetylbleomycin A_2; PLM, phleomycin; TLM, tallysomycin; EBSS, Earle's basal salt solution; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)-aminomethane; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis[2-(5-phenyloxazolyl)]benzene.

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