Invited Review

Bleomycin: New Perspectives on the Mechanism of Action[1]

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The bleomycin group antitumor antibiotics have long been of interest as a consequence of their efficacy in the treatment of certain tumors, not to mention their unique structures and properties in mediating dioxygen activation and sequence selective degradation of DNA. At a chemical level, the structure originally assigned to bleomycin was subsequently reassigned and the new structure has been confirmed by total synthesis. Through the elaboration of structurally modified bleomycin congeners and fragments, synthetic efforts have also facilitated an understanding of the contribution of individual structural domains in bleomycin to sequence selective DNA binding and cleavage, and have also provided insights into the nature of the chemical processes by which DNA degradation takes place. Within the last several years, it has also become apparent that bleomycin can mediate the oxidative degradation of all major classes of cellular RNAs; it seems entirely plausible that RNA may also represent an important locus of action for this class of antitumor agent. In parallel with ongoing synthetic and mechanistic efforts using classical methods, the study of bleomycins attached to solid supports has been shown to provide important mechanistic insights, and the actual elaboration of modified bleomycins by solid phase synthesis constitutes a logical extension of such efforts.

Introduction

Since their discovery by Umezawa and co-workers as a family of antitumor antibiotics elaborated by *Streptomyces verticillus*, the bleomycins have been the focus of detailed structural, biosynthetic, synthetic, mechanistic, and therapeutic investigations. They are presently employed clinically in combination with a number of other agents for the treatment of several types of tumors, notably squamous cell carcinomas and malignant lymphomas.[2] The bleomycins are commonly employed therapeutically as a mixture of several congeners denoted blenoxane, which consists predominantly of bleomycin A_2 and bleomycin B_2.[3] The efficacy of bleomycin as an antitumor agent has been established, for example, by the finding that omission of bleomycin from a multidrug regimen employed for the treatment of germ cell carcinomas resulted in a substantial lessening of efficacy.[4]

In this review, I have emphasized structural and mechanistic studies of the bleomycins that include the focus of investigations by my laboratory and which may well hold the key for defining new strategies for antitumor therapy.

Structural and Synthetic Studies. In common with the structurally related phleomycins,[5] early studies revealed that the bleomycins (BLMs) were biosynthesized as a mixture of congeners separable by paper and ion-exchange chromatography.[6] The congeners proved to differ in structure solely at the C-terminus. Because the biosynthesis proceeds from the N- to C-terminus, it has also been found possible to induce the biosynthesis of numerous structural analogues of bleomycin differing only at the C-terminus by inclusion of individual amines in the fermentation media employed for BLM-producing strains of *Streptomyces*.[3,7]

While most of the naturally occurring BLMs differ only at the C-terminus, there have been reports of a number of other structurally related species that differ from bleomycin elsewhere within the structure.[8,9] Perhaps the best characterized of these are the tallysomycins,[9] which differ from the bleomycins structurally in at least three ways. As shown in Figure 1, this includes the absence of an α-CH_3 group in the valerate moiety of tallysomycin, the presence of a talose sugar as part of a structurally unique glycosylcarbinolamide functionality, and the presence of C-substituents, most of which are different from those observed for the bleomycins and phleomycins.[9]

The structures of the bleomycin group antibiotics have been determined following chemical degradation, e.g., by hydrolysis of the amides in bleomycin. The hydrolysis products so obtained have been prepared by chemical synthesis; each has now been elaborated using a few different strategies. This approach has provided building blocks that were utilized by the Hecht and Umezawa laboratories to effect total syntheses of bleomycin, both of which were reported in 1982.[10,11] Improvements in synthetic methodology have permitted both laboratories to improve the ease and efficiency of their original syntheses.[12,13] The Boger laboratory has also explored the chemistry of bleomycin, reporting a total synthesis in 1994.[14]

A number of benefits have accrued from the efforts in chemical synthesis. The structure originally proposed for bleomycin was incorrect; it posited the involvement of the propionate moiety of the N-terminus of bleomycin in a β-lactam structure with N^{β} of the β-aminoalanineamide.[15] The reassigned structure[16] has been confirmed through synthetic efforts. Also established in part by synthesis has been the stereochemistry of several of the 19 asymmetric centers in BLM. An example which can be cited from efforts in the Hecht laboratory involved the synthesis of *S-erythro*-β-hydroxyhistidine from glucosamine; the stereo centers at

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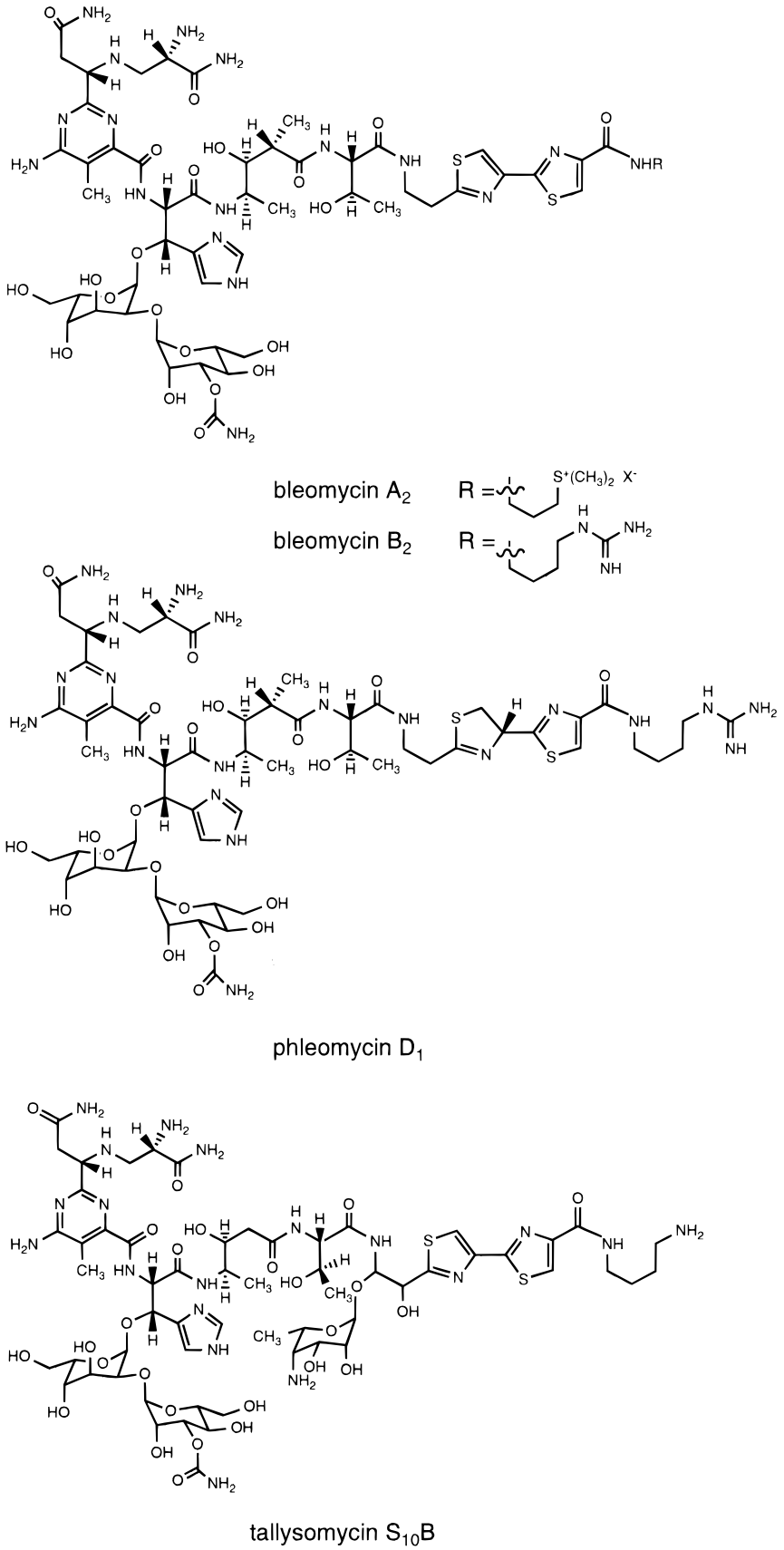
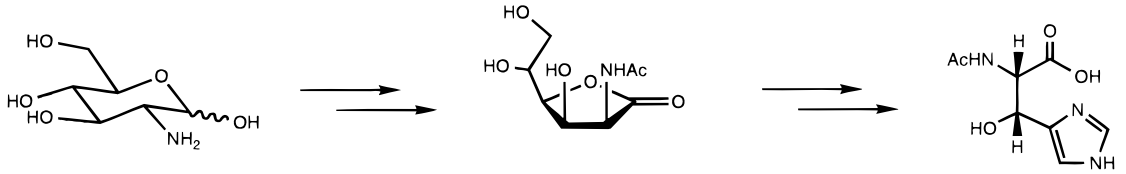


Figure 1. Structural formulas of bleomycin A_2 and B_2, phleomycin D_1, and tallysomycin $S_{10}B$.

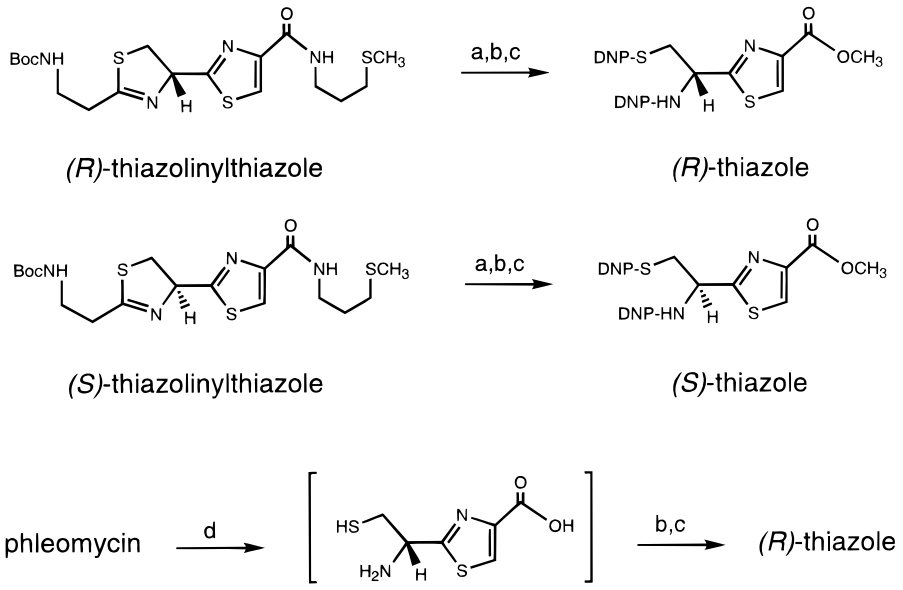
C-2 and C-3 in the sugar unambiguously defined the stereochemistry of the derived histidine derivative (Scheme 1).[17]

Also investigated has been the stereochemistry of phleomycin. As is clear from Figure 1, phleomycin has a thiazinylthiazole moiety in lieu of the bithiazole present in BLM. Because the biosynthesis is believed to involve dehydrative cyclization of cysteinyl peptides, followed by hydrogenation

Scheme 1. Synthesis of *S-erythro-β*-Hydroxyhistidine from Glucosamine



Scheme 2. Degradation of Phleomycin To Obtain a Chiral Fragment of the Thiazolinylthiazole Moiety, Which Was Compared with the Products of Analogous Degradation of Two Authentic Thiazolinylthiazoles Prepared by Umambiguous Synthesis[a]



[a] (a) 4.5 N HCl; (b) 2,4-dinitrofluorobenzene; (c) CH_2N_2; (d) 5.5 N HCl.

of the formed thiazolines to afford thiazoles, phleomycin apparently accumulates in strains of *Streptomyces* that fail to effect the dehydrogenation of one thiazoline.[18] That BLM and PLM have identical stereochemistry at the 19 asymmetric centers that they share in common has been shown by dehydrogenation of PLM chemically to afford a species indistinguishable from naturally derived BLM. As outlined in Scheme 2, we have shown that the stereochemistry of the asymmetric centers in the thiazoline moiety of PLM is *R*.[19] This finding is important to an understanding of the way in which phleomycin binds to DNA.

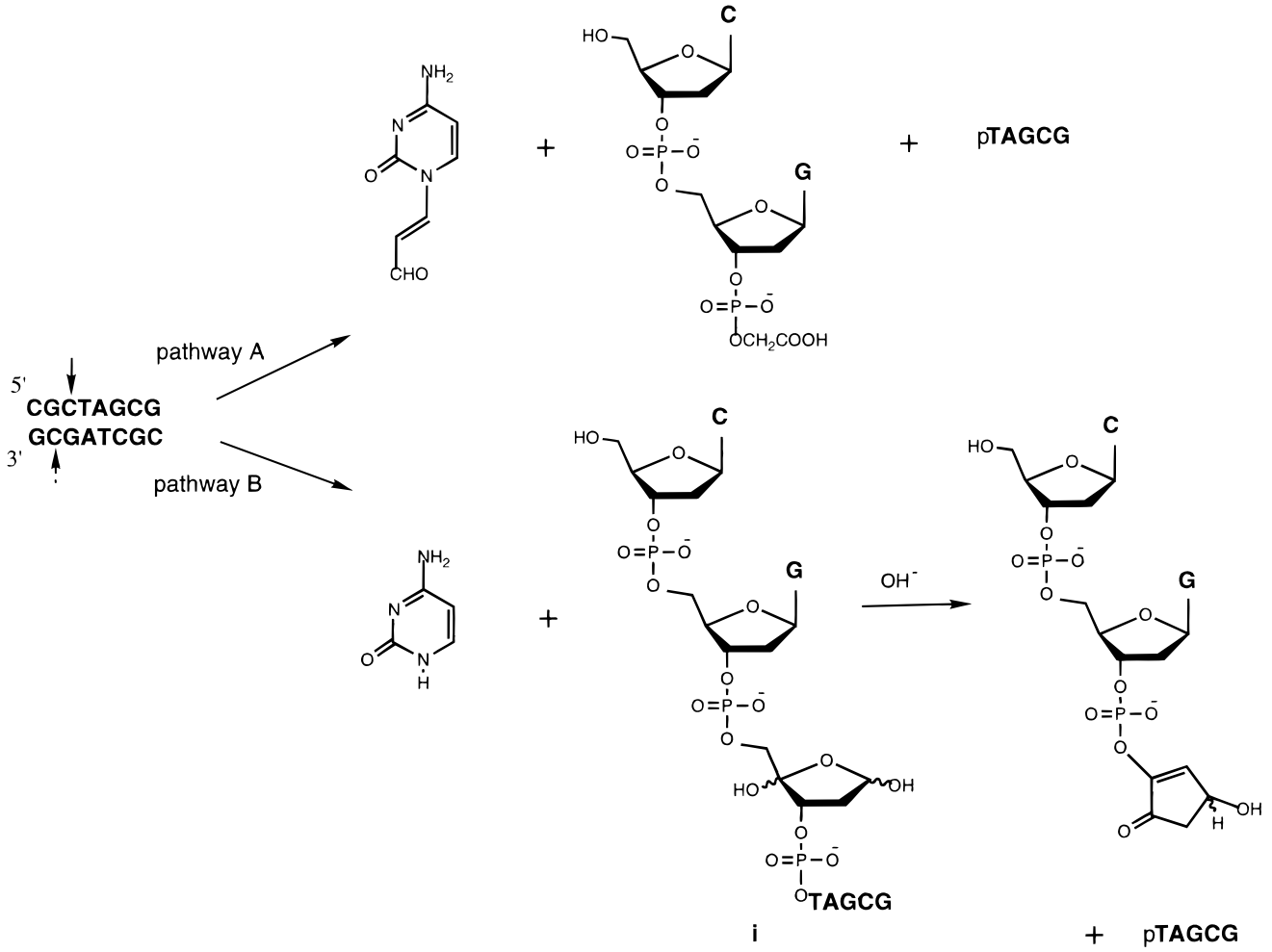
At present, much less is known definitively about the stereochemistry of tallysomycin. On the basis of its structural similarity to BLM and PLM, it seems likely on biosynthetic grounds that the asymmetric centers that they share in common have the same stereochemistry, a proposal that is supported by a recent NMR study of Zn(II)·tallysomycin.[20] Likewise, the stereochemistry of the talose moiety has been established, but not that of the two C atoms bearing oxygen within the aminoethylbithiazole moiety.

As described below, the study of the chemistry of BLM and its fragments and analogues has also afforded a number of insights into the chemical behavior of the molecule that have important consequences for its mechanism of action.

The Chemistry of Bleomycin Oxidation and Oxygenation Reactions. Certainly the best characterized property of BLM is its ability to effect the degradation of DNA substrates. This has been established utilizing a number of DNA substrates ranging from chromatin in intact eukaryotic cells[21] to mixtures of isolated DNAs and plasmids in cell free systems.[22] At the level of chemical characterization of products, perhaps the most interesting substrates have been DNA oligonucleotide substrates of known and uniform sequence. These have included restriction endonuclease digest of plasmid DNAs, which have permitted definition of the sequence selectivity of DNA cleavage by bleomycin[23] as well as short synthetically prepared DNA oligonucleotides, the latter of which have facilitated characterization of the actual chemical products resulting from degradation of DNA by bleomycin.[24]

Bleomycin-mediated DNA degradation requires the presence of a redox-active metal ion such as Fe^{2+} or Cu^{+}, as well as molecular oxygen.[22] As illustrated in Scheme 3 using the self-complementary DNA octanucleotide $^{5'}$CGCTAGCG$^{3'}$ as a substrate, bleomycin produces two sets of products from B-form DNA substrates. One of these (pathway A) results in frank DNA strand scission,[24] while the other (pathway B) affords base release at the site of the BLM-induced lesion with concomitant formation of a 4′-hydroxyapurinic acid moiety (**i**, the "alkali-labile" lesion).[25] The latter intermediate can be induced to undergo DNA strand scission by admixture of any of a few different reagents, including alkali,[25] alkylamines,[26] or hydrazine.[27] It may be noted that both sets of products derive from a common intermediate, namely, an initially formed C-4′ radical resulting from the abstraction of a H atom from the DNA substrate by bleomycin. This radical intermediate can combine with dioxygen, forming a hydroperoxy radical; fragmentation of the oxygenated sugars via a Criegee-type process then affords a base propenal and an oligonucleotide terminating in a 3′-phosphoroglycolate moiety (Scheme 3). Alternatively, the sugar radical can undergo oxidation, perhaps mediated by bleomycin itself, to form a carbocation that reacts with water to afford the alkali-labile lesion. Thus, both products may be regarded as oxidation products of DNA.

Scheme 3. Products Resulting from Degradation of a Self-Complementary Oligonucleotide by Bleomycin[a]



[a] Only the products resulting from modification at deoxycytidine$_3$ are shown.

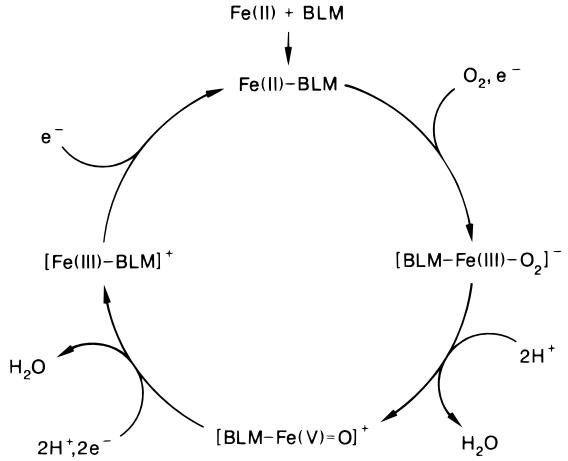


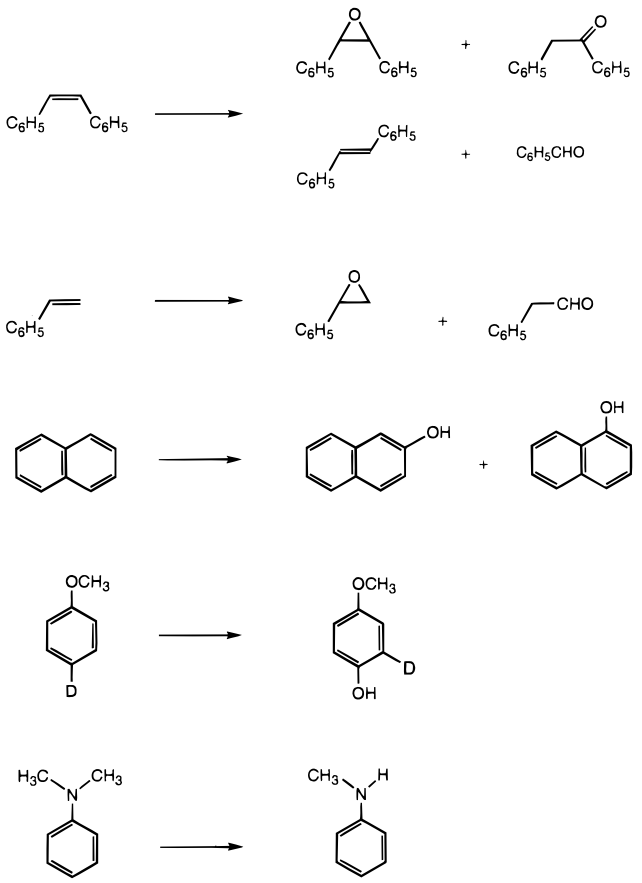
Figure 2. Possible catalytic cycle for Fe•bleomycin.

It is interesting that the same products can be formed from DNA when BLM is activated with $Fe^{3+} + H_2O_2$, rather than $Fe^{2+} + O_2$.[28] This has led to the suggestion that BLM activation for DNA cleavage involves the formation of a BLM−metal hydroperoxide intermediate that undergoes heterolytic O−O cleavage either prior to or concomitant with DNA oxidation.[22,29] A possible catalytic cycle is suggested in Figure 2. In fact, the catalytic cycle outlined in the figure is strongly reminiscent of the activation of cyctochrome P450 and its model compounds, the latter of which are capable of mediating both oxidation and oxygenation of low molecular weight substrates.[30]

The validity of the analogy between the chemistry of activated BLM and that of cytochrome P450 is supported by the remarkable similarity of the chemistry that these species mediate when low molecular weight compounds are employed as substrates; some examples of the chemical transformations mediated by bleomycin are shown in Scheme 4.[31]

Unlike the chemistry of small molecule oxidation/oxygenation, which seems likely to occur by bimolecular collision of activated BLM with its substrates, the oxidative transformation of DNA substrates by bleomycin seems to involve at least two steps, namely, substrate binding and H atom abstraction. Bleomycin has been noted to effect DNA degradation in a sequence selective fashion at a subset of $^{5'}$GC$^{3'}$ and $^{5'}$GT$^{3'}$ sites. This selectivity may result from the binding of metallobleomycins with enhanced efficiency to certain sites in DNA, to variations in DNA microstructure which render certain C-4′ H atoms more readily amenable to abstraction, or both. The importance of the H abstraction step in contributing to the observed sequence selectivity of cleavage by BLM is underscored by the finding that H abstraction from different DNA sequences is associated with different isotope effects.[32] H atom abstraction seems to be rate-limiting for DNA degradation; it appears likely that the facility of H atom abstraction from a given site is an important determinant of the extent of cleavage at that site, especially under

Scheme 4. Oxidative Transformations of Low Molecular Weight Substrates by Fe·Bleomycin



experimental conditions that involve cleavage at a limited number of DNA sites.

While DNA cleavage clearly cannot occur at sites not bound by BLM, it is known from studies with Co·BLM that Co·BLM binds efficiently only at certain sites and that cleavage does not occur at all bound sites.[33] Thus, sequence selectivity of cleavage must depend both on binding efficiency and the facility of H abstraction at a given site.

Functional Domains of Bleomycin. As shown in Figure 1, bleomycin contains at least four functional domains. These include the metal-binding domain, which is responsible for metal ion binding and O_2 activation and which must ultimately mediate the abstraction of H atoms from the DNA substrate.[22,34] The bithiazole and C-terminal substituent are known to be involved in DNA binding; removal of the C-terminal substituent (to afford bleomycinic acid)[3] or introduction of a substituent that lacks a positive charge under the conditions of DNA cleavage dramatically diminishes the efficiency of DNA cleavage by bleomycin.[35] Ohno and co-workers first demonstrated the importance of the linker region between the metal-binding domain and bithiazole moieties to the efficiency of DNA cleavage by bleomycin,[36] and the Boger laboratory has contributed importantly to our understanding of the function of this structural element.[37] The least well characterized of the functional domains of bleomycin at present is the carbohydrate moiety; this domain seems likely to participate in cell recognition by bleomycin and possibly in cellular uptake and metal ion coordination.[22]

As regards the metal-binding domain, numerous studies have documented the ability of this portion of the BLM molecule to bind numerous metal ions, although the specific atoms involved as ligands have sometimes been controversial.[20,34,38] A more surprising finding is that the metal-binding domain also constitutes the primary determinant of the sequence selectivity of DNA cleavage by BLM. Evidence in support of this conclusion includes the finding that the alteration of the C-terminal substituent of BLM had no effect on the strand selectivity of cleavage at a high-efficiency DNA cleavage site, while alteration of the metal-binding domain exhibited dramatic effects in altering strand selectivity.[39] A second line of evidence was provided by Mascharak and co-workers, who demonstrated that a preformed Fe(III) complex of a ligand structurally related to the metal-binding domain of BLM gave a DNA cleavage pattern virtually indistinguishable from that of Fe(III)·BLM itself when each was activated with H_2O_2.[40] Presumably, the stoichiometric activation of this molecule from a preformed Fe(III) complex must compensate for the diminished DNA affinity resulting from the absence of the DNA-binding domain.

One further line of evidence was obtained in the Hecht laboratory using the deglycoBLM analogues shown in Figure 3. These contained the normal metal-binding and bithiazole domains, but had variable numbers of glycine residues ($n = 0$, 1, 2, or 4) in place of threonine. Assuming that increasing numbers of glycine residues would cause the metal-binding and DNA-binding domains to be separated by increasing distances when bound to DNA, it seemed inevitable that one of these two domains would be forced to bind to DNA substrates at sites increasingly distant from the normal binding site. DNA cleavage experiments revealed that the site of DNA cleavage was unaltered, indicating that the metal-binding domain must be the primary determinant of the site of DNA binding and cleavage by BLM.[41] While the original experiments employed deglycoBLM analogues that lacked any positive charge with the C-terminal substituent, subsequently those experiments have been repeated using analogues of deglycoBLM A_2 and afforded the same results (S. Kane, A. Natrajan, and S. M. Hecht, unpublished).

Recently, the Hecht laboratory has prepared a number of chlorinated derivatives of deglycoBLM and of the bithiazole moiety of BLM (Figure 4).[42] In the presence of Fe^{2+} + O_2, these deglycoBLM derivatives effected cleavage of d(CGCTAGCG) at the GC sequences in precisely the same fashion as deglycoBLM and BLM. However, when photolyzed, these derivatives underwent homolysis of the C−Cl bond and caused cleavage of the DNA oligonucleotide at the T and A residues of the octanucleotide. Remarkably, the chlorinated bithiazoles gave precisely the same pattern of light-dependent cleavage of this DNA substrate as the chlorinated deglycoBLMs. This finding indicates that both the metal-binding domain and bithiazole + C-terminal domain of BLM have preferred DNA-binding sequences. It seems likely that a hallmark of DNA sequences that are bound and cleaved efficiently by BLM is the presence of adjacent sequences that permit both of these structural domains to bind to their own preferred sequences simultaneously.

Several NMR studies of metalloBLMs have indicated that these species assume a bent conformation when they bind to DNA.[43] These findings argue that there must be a preferred conformation within the valerate moiety of the linker region, which constitutes the locus at which the bend is localized. This conclusion is strongly supported by studies carried out in the Boger laboratory, which have involved systematic alteration of the structure of the linker region.[37] In fact, the efficiency of DNA cleavage by these derivatives

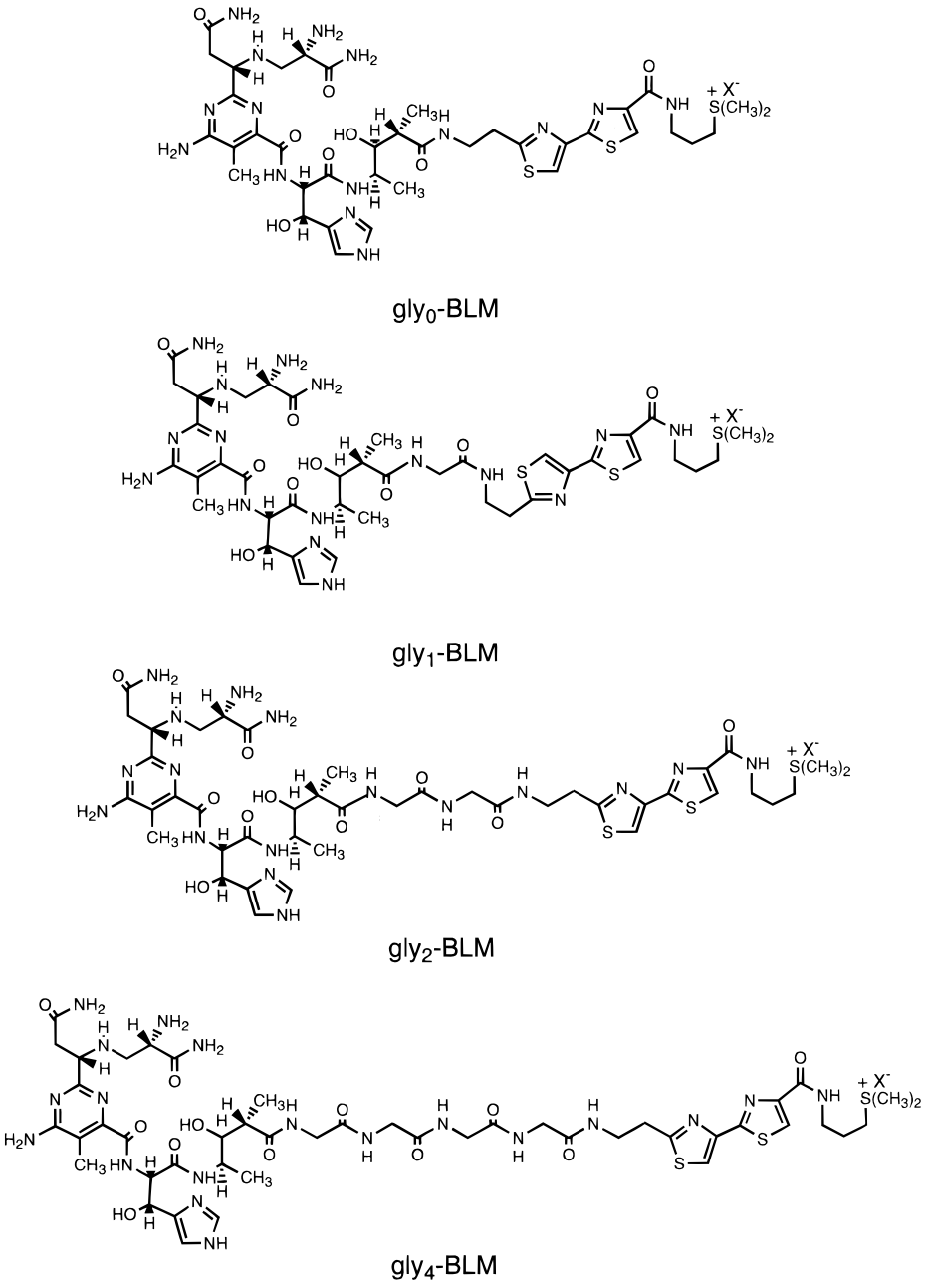


Figure 3. DeglycoBLM analogues having variable numbers of glycine residues in place of threonine.

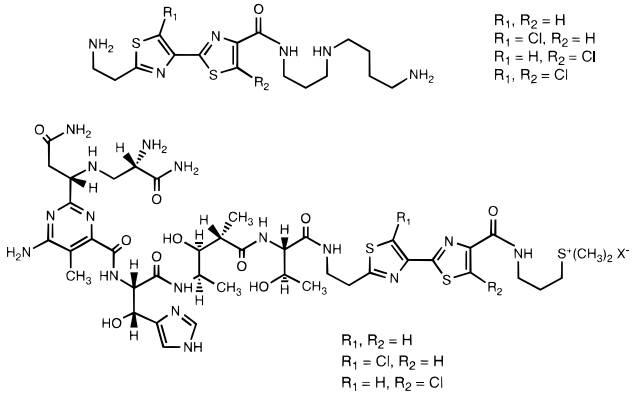


Figure 4. Chlorinated derivatives of deglycoBLM and the bithiazole moiety of BLM.

can be rationalized by their ability to assume the optimal conformation required for binding of the DNA substrate.

Effect of DNA Structure Modification on Cleavage by BLM. As is clear from the foregoing discussion, much of our present understanding of the action of bleomycin has resulted from the use of analogues of BLM or of its functional domains. A complementary approach has involved the use of DNA substrates whose structures differ from those of B-form DNA.

The DNAs studied have included Z-DNA, which is not a good substrate for bleomycin,[44] and B-form DNAs modified in the minor and major grooves. Thus, alteration of the minor groove by modification of guanosines with aflatoxin strongly inhibited DNA oligonucleotide cleavage by BLM at $^{5'}GC^{3'}$ and $^{5'}GT^{3'}$ sites,[45,46] as did admixture of the minor groove binder distamycin in addition to BLM.[47] In contrast, glycosylated DNA, in which the major groove contained numerous carbohydrate residues, was essentially unaltered in DNA cleavage specificity by BLM.[44,46] These observations strongly support the view that BLM binds to DNA in the minor groove.

Of particular interest are the effects of distorting B-DNA

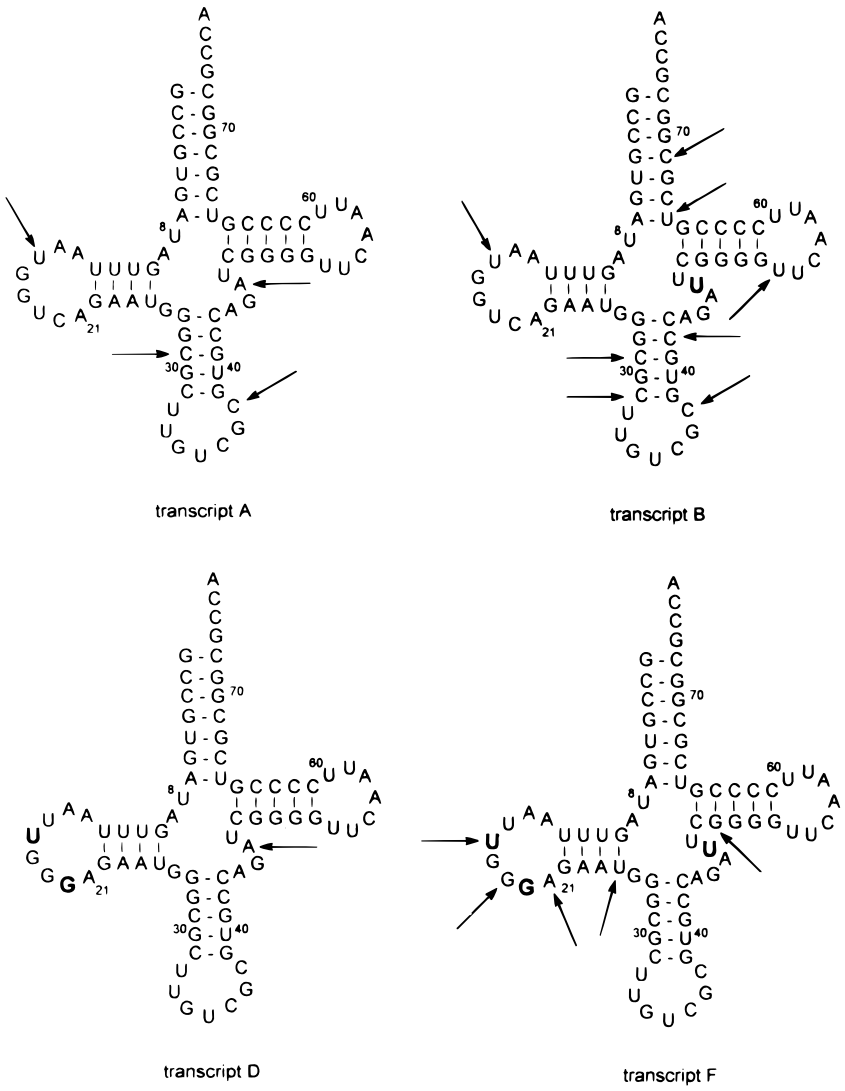


Figure 5. Four tRNA transcripts of related sequence cleaved at different positions (arrows) by Fe(II)·BLM.

structure on the cleavage specificity by BLM. Changes as simple as DNA methylation, which has been found to alter DNA conformation,[48] resulted in a significant change in the efficiency of cleavage at $^{5'}$G·pyr$^{3'}$ sites as far as 14 base pairs from the site of DNA strand scission[49] and also affected the distribution of cleavage products.[50] More pronounced structural changes, e.g., those associated with DNA platination[51] or the introduction of DNA bulges,[52] did not eliminate DNA cleavage but resulted in a change in the specificity of DNA cleavage. Thus the preference for cleavage at $^{5'}$GC$^{3'}$ and $^{5'}$GT$^{3'}$ sites is obtained only when these sequences are present in the context of a B-DNA structure. This argues that metalloBLMs recognize the shape of their DNA substrates and that $^{5'}$G·pyr$^{3'}$ sequences have a shape that is optimal for BLM binding and cleavage. In this context, it is worthy of note that the $^{5'}$G·pyr$^{3'}$ sequences correspond to B-DNA structures having the widest, shallowest minor grooves. Given that all metallo·BLMs studied to date by ^{1}H NMR have been found to have metal-binding domains at least as large as the width of the minor groove,[43] cleavage at $^{5'}$G·pyr$^{3'}$ sequences may simply reflect the sites at which the C-4′ H atoms are most accessible for abstraction by BLM. This suggestion is also consistent with the highly specific patterns of cleavage of DNA at a duplex−triplex junction, a type of structure in which the width of the minor groove is believed to change substantially over the distance of a few nucleotides (Figure 5).[53] In these distorted B-form DNA structures, it is clearly DNA shape rather than sequence that determines the site(s) of cleavage.

That the nature of DNA cleavage by BLM per se has not changed in these distorted structures may be judged from the actual chemistry of DNA cleavage. In none of the cases discussed above has any new DNA cleavage product been noted, although as noted above, the ratio of strand scission products versus alkali labile lesion can be altered somewhat in the case of methylated DNAs (cf., Scheme 3).[50]

Cleavage of RNA by Bleomycin. While early studies of RNA−BLM interaction failed to provide any evidence of RNA degradation, in 1989 Magliozzo et al. demonstrated that 0.3 mM Fe(II)·bleomycin effected limited degradation of several different tRNAs.[54] Subsequent studies carried out in the Hecht laboratory using a number of tRNAs and tRNA precursor transcripts revealed that there was an enormous range of responses to treatment with Fe(II)·BLM. Some of these RNAs, such as *B. subtilis* tRNA^{His} precursor transcript, were cleaved with efficiencies comparable to that of B-DNA, albeit only at a limited number of sites, while others such as the *E. coli* tRNA^{Tyr} precursor transcript were not cleaved to any significant extent even at much higher BLM concentrations.[55]

Further, the patterns of cleavage for those tRNAs that were substrates for degradation by BLM differed somewhat from the pattern of cleavage observed for B-DNA. While

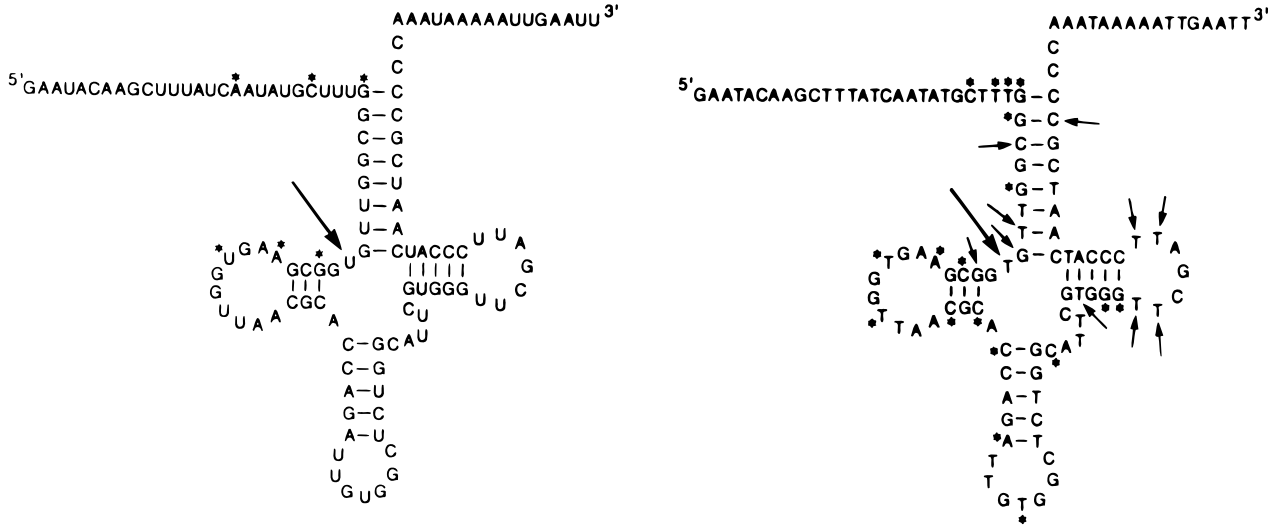


Figure 6. Cleavage of *B. subtilis* $tRNA^{His}$ precursor and a "tDNA" transcript having the same primary sequence by Fe·BLM. Strong sites of cleavage are indicated by large arrows, lesser intensity cleavage sites by small arrows, and minor sites by asterisks.

$^{5'}$G·pyr$^{3'}$ sites were prominent among those sites cleaved by Fe·BLM, a disproportionate number of cleavage sites were located at the junctions between single- and double-stranded regions in the tRNA substrates.[56] Also, even small changes in tRNA structure could result in substantial alterations in the pattern of tRNA cleavage (Figure 5).[57] Thus, in common with DNA, tRNA conformation must play an important role in determining those sites susceptible to cleavage by BLM.

DNA oligonucleotides having the same nucleotide sequences as tRNAs have been studied previously. These have been shown to have conformations most similar to those of the corresponding tRNAs, as judged by their utilization as substrates by enzymes that normally use the tRNAs as substrates.[58] To further define the structural parameters that render polynucleotides susceptible to cleavage by Fe·BLM, it seemed logical to use a tDNA as a substrate for cleavage by Fe·BLM, in direct comparison with the corresponding tRNA.[59] As shown in Figure 6, $tRNA^{His}$ precursor transcript and $tDNA^{His}$ were cleaved at the same major site, again arguing that preferred sites of cleavage are determined by the specific conformations of the substrates. Given the results summarized in Figure 5, which illustrate the sensitivity of BLM as a probe for RNA conformation, the similarities in tRNA and tDNA cleavage shown in Figure 6 are that much more striking.

However, there are also clear differences in tRNA and tDNA cleavage evident from Figure 6, notably in the relative extents of cleavage at position 35 and the selectivity of cleavage at that site. Clearly, cleavage occurs to a lesser extent and with greater selectivity for the tRNA. This was shown by competition experiments not to be due to the strength of substrate binding by Fe·BLM; this metallo-BLM clearly binds more avidly to $tRNA^{His}$ than to the corresponding tDNA. Two possibilities were suggested to rationalize this observation. One possibility suggested was that RNA produces damage that does not lead to RNA strand scission; the other was that the binding of BLM to RNA occurs in an orientation not conducive to the production of strand breaks.[60] In fact, it has subsequently been suggested by David Crich and a co-worker that radicals formed from RNA sugars may well be longer lived than those derived from DNA and may actually be "repaired" by H abstraction, thus limiting the consequences of radical formation in RNA.[61] Further, using a chimeric oligonucleotide containing a single ribonucleotide within a DNA oligomer, we have obtained evidence that such species undergo oxidation at both the C-4′ and C-1′ positions of ribose and that the chemistry of oligonucleotide degradation is more complex than that of DNA itself.[62] This finding was underscored in a more recent study using the same chimeric substrate, which suggested the presence of structurally modified intermediates which are analogous to the alkali-labile lesion formed from DNA by the action of BLM in that they fail to undergo frank strand scission following treatment with BLM.[63]

In addition to effecting the strand scission of tRNAs and tRNA precursor transcripts, BLM has also been shown to be capable of mediating strand scission of messenger[64] and ribosomal RNAs,[56] as well as the RNA strand of an RNA−DNA heteroduplex.[65] Again, cleavage was highly selective, involving oxidative transformation of a small number of sites relative to what is normally observed for B-DNA substrates.

RNA as Locus of Antitumor Action. While early studies of the bleomycin mechanism of action considered a number of possible loci of action for the drug, including DNA and RNA polymerases and nucleases, as well as DNA ligase,[66] the finding of extensive DNA degradation by metalloBLMs both in vitro and in vivo has tended to focus experimental efforts on the characterization of BLM-mediated DNA degradation. In early studies, efforts to demonstrate RNA binding and cleavage were unsuccessful, perhaps due to a lack of appreciation that metal ions are required for the action of bleomycin. Nonetheless, the early lack of success resulted in a paucity of studies of RNA as a potential locus of action for bleomycin.

If one considers the evidence in support of DNA cleavage as a mechanism for expression of the antitumor activity of bleomycin, critical observations include the ability of bleomycin to effect facile and extensive degradation of chromatin.[35] The fact that BLM can produce both single- and double-strand breaks can be argued to support the importance of DNA cleavage as a basis for the therapeutic action of BLM, as can the fact that extensive DNA damage will inevitably lead to diminished clonigenic potential. On the other hand, BLM-mediated DNA damage can also be repaired with facility,[35] and the concentration of BLM required to compromise clonigenic potential in cultured cells seems quite high relative to what is likely to be achieved following the clinical administration of 7−8 mg (∼5 μmol) of blenoxane in a clinical setting. The recent

findings that inhibitors of DNA polymerase β can potentiate the toxicity of BLM toward cultured P388D_1 cells[67] is consistent with the interpretation that the cytotoxicity of BLM can be mediated at the level of DNA damage, but does not prove that the cytotoxic response is due to DNA damage in the absence of such inhibitors. There have also been suggestions of possible limitations in the ability of BLM to cross the cellular and nuclear membranes efficiently,[68] which would limit access to the DNA target in a therapeutic setting. It has also been shown that BLM preferentially damages DNA in active genes and in the linker region of the nucleosome.[69]

In comparison, access to RNAs should be more facile since many RNAs are present in the cell cytoplasm and are not thought to be packed intensively. Further, since there seems to be limited evidence for the repair of damaged RNAs, it is attractive to imagine that destruction of a key cellular RNA could be the mechanism by which BLM destroys tumor cells. In this context, it is important to consider examples of RNA targeting that lead to loss of cell viability. These may be thought to include the aminoglycoside and erythromycin classes of antibiotics, which target ribosomal RNAs in bacteria,[70] as well as cytotoxic proteins such as ricin, which effect mammalian cell killing via rRNA depurination.[71] A further example that may be cited is onconase, a cytotoxic member of the RNase superfamily, which exerts its antitumor effects at the level of tRNA degradation.[72]

Bleomycin Conjugates: A Tool for Dissection of Mechanism. New opportunities in studies of the mechanism of action of bleomycin have resulted from the finding that immobilization of BLM on a solid support by covalent linkage through the C-terminal substituent has little effect on the ability of metallobleomycins to cleave DNA.[73] The initial observations involved the conjugation of BLM A_5 to a controlled pore glass bead that had been functionalized with a glycolic acid linker. The immobilized Fe(II)·BLM effected relaxation of plasmid DNA and cleavage of a 5′-^{32}P end-labeled DNA restriction fragment. Surprisingly, the efficiency of DNA cleavage was only about 5-fold lower than that of free Fe(II)·BLM. Moreover, the sequence selectivity of DNA cleavage was identical with that of free DNA, and neither the extent nor sequence selectivity of DNA cleavage was significantly affected by the nature of the solid support, nor the length of the tether connecting the BLM to the solid support (A. T. Abraham, X. Zhou, and S. M. Hecht, unpublished results). As regards the potency of cleavage, it is possible that conjugation of BLM to a solid support actually does reduce its potency as a DNA-cleaving agent. However, it is perhaps more likely that diminution of cleavage results from localization of BLM to a solid support whose concentration in the reaction mixture is 10^{12}-fold lower than that of BLM itself.

From the perspective of the mechanism of DNA cleavage, there are important consequences of these observations. Because the C-terminal substituent in the BLM conjugates includes a glass bead that is $\sim 10^5$ times larger than the remainder of the BLM molecule (and much larger than the DNA oligonucleotide substrate as well), it seems inconceivable that this substituent could undergo threading intercalation. Since the modeling studies reported to date also indicate that the metal-binding domain is at least as large as the DNA minor groove,[43] this end of the molecule is also unlikely to pass through the helix, especially since separation of the strands is known to be energetically unfavorable.[74]

To further diminish the chances that the observed cleavage could have involved denaturation of the DNA sufficient to form a single-stranded DNA, which then rehybridized around the BLM conjugate, the cleavage experiment was repeated using a relaxed circular DNA duplex, i.e., a substrate lacking ends. In this experiment, pBR322 plasmid DNA was admixed with Fe(II)·BLM A_5-CPG, and the reaction was permitted to proceed until there was an average of <1 nick per plasmid. The plasmid was then treated with restriction endonucleases *Hin*dIII and *Eco*RV, and the resulting 158-base pair duplex was 5′-^{32}P end-labeled and analyzed on a polyacrylamide gel to define the position(s) of cleavage. In fact, the major position of cleavage was identical with that mediated by free Fe(II)·BLM A_5. Thus, cleavage of DNA by Fe·BLM can occur without the need for threading intercalation of the bithiazole moiety (A. T. Abraham, X. Zhou, and S. M. Hecht, unpublished results).

Repetition of these experiments in the presence of conjugated Co(III)·BLM A_5 or Cu(II)·BLM A_5 + a reductant also gave DNA cleavage qualitatively indistinguishable from the free metallobleomycins. In both cases, the metalloBLM conjugates were only slightly less potent than the free metallobleomycins. Thus, it would seem that none of the three best characterized BLMs require threading intercalation as a prerequisite to DNA cleavage.

Future Prospects. Although the study of the bleomycins has now been underway for more than thirty years, many fundamental issues remain to be addressed. These may be thought to include better definition of the relative importance of DNA and RNA as therapeutic targets for bleomycin and an enhanced understanding of the nature of the molecular interaction of bleomycin with these macromolecules. Also critical is the development of an understanding of the structural features in bleomycin that facilitate tumor cell recognition and the mechanism(s) of cellular uptake, distribution, and ultimate export or conversion to authentically inactive metabolites. The identification of inhibitors of polymerase β may lead to the development of compounds capable of potentiating the antitumor action of bleomycin, by blocking repair of the damage that it inflicts on tumor cells. Finally, the availability of methods of combinatorial synthesis suggests that it may be possible to create libraries of bleomycin congeners from which agents with improved properties could be selected.

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References and Notes

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