Bleomycins: A Structural Model for Specificity, Binding, and Double Strand Cleavage

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The bleomycins (BLMs; Figure 1) are a class of glycopeptide antibiotics isolated from *Streptomyces*.[1] They have found clinical use in combination chemotherapy for the treatment of head and neck cancer, certain lymphomas, and testicular cancer.[2] The mechanism of cytotoxicity is thought to be related to the ability of BLMs to bind to and degrade duplex DNA in the presence of Fe^{2+}, O_2, and a reductant as required cofactors.[3a] Under *in vitro* conditions, BLM causes both single strand (ss) and double strand (ds) breaks in DNA. The ratio of these two cleavage processes is sequence-dependent, with ds cleavage being a relatively minor event.[4] *In vivo*, ssDNA cleavage is probably repaired by the human counterparts of prokaryotic DNA repair enzymes.[5] The less frequent dsDNA cleavage, however, is believed to be responsible for the observed cytotoxicity of BLM, and its mechanism of repair has not thus far been elucidated.

A unified hypothesis explaining the basis of BLM−DNA recognition and the chemical specificity of cleavage has been an important goal of the field. Beginning with the seminal experiments of Peisach, Horwitz, and Burger nearly 20 years ago on the metal and oxygen requirements of BLM,[3,6] a general consensus has emerged concerning the mechanistic details of the ssDNA cleavage. These studies have been summarized in a number of recent reviews.[7,8] However, despite the efforts of many groups, the structural basis for the sequence-specific cleavage of DNA by BLM and the mechanistic basis for dsDNA cleavage have remained elusive. This brief review will focus on these two topics. First, the very recent 2D NMR studies of an analog of "activated BLM", a cobalt(III) hydroperoxide (HOO−CoBLM), bound to a specific GpPy binding site in an oligonucleotide will be presented. These studies reveal the basis for sequence selectivity of cleavage and the mode of binding. The relationship of these studies to activated FeBLM's structure and function will be discussed. Second, a chemical and structural model will be presented to provide a working hypothesis of how a single molecule of BLM can mediate dsDNA cleavage. Taken together this recent work provides a unifying context for understanding this remarkable class of DNA cleaving agents.

Background

The BLMs bind a variety of metals. In the presence of an oxidant, a number of metallo-BLMs can effect DNA strand scission. Most effort has focused on understanding the iron-BLMs. However, the concentration of free copper in cells and the ability of copper-BLMs to mediate strand scission have generated some interest in these complexes as well.[7,9,10] Using a variety of time-resolved biophysical methods, the

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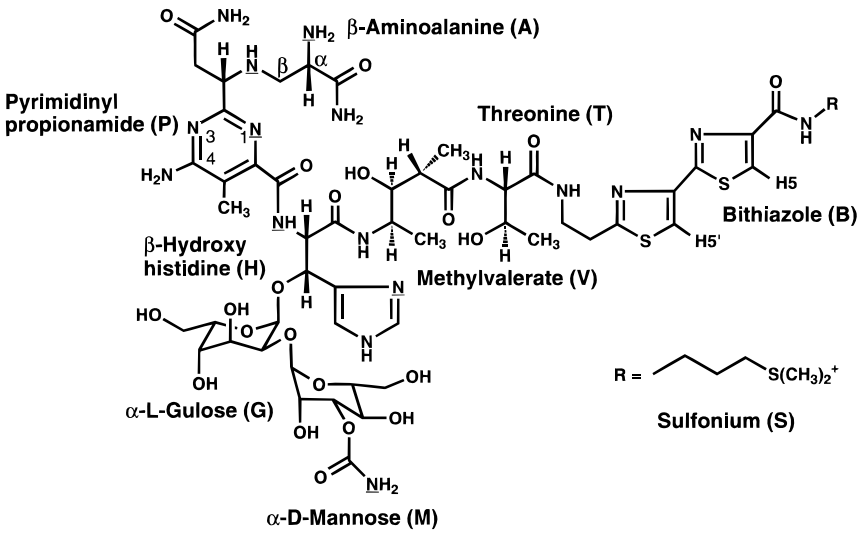
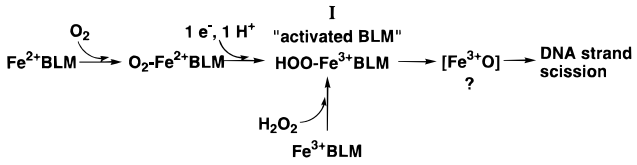


Figure 1. Structure of bleomycin A2. Nitrogen ligands coordinating to the metal are underlined.

Scheme 1



Peisach and Horwitz laboratories definitively established the sequence of events (Scheme 1) leading to an activated BLM with DNA cleaving capabilities.[6]

Intermediate **I**, designated activated BLM, has been identified recently by electrospray mass spectrometry as a ferric hydroperoxide and is the last detectable intermediate prior to DNA strand scission.[11] Remarkably, this intermediate has a half-life of 2 min at 4 °C.[6a] Whether the peroxide itself initiates DNA cleavage or is broken down via heterolysis of the O−O bond to an iron oxene equivalent, [Fe^{3+}O], which initiates DNA cleavage, is the subject of ongoing investigations. Recent circular dichroism, magnetic circular dichroism, and X-ray absorption studies have suggested that a key to the unique chemical reactivity of iron-BLM resides in one of its equatorial ligands, the pyrimidine moiety (P; Figure 1).[12a] Strong back-bonding from the iron to this ligand reduces the charge transfer between the iron and O_2 and thus decreases the facility of the ternary O_2−Fe^{2+}BLM complex to decompose to $O_2^{\bullet-}$ and Fe^{3+}BLM. A further understanding of the chemistry of the non-heme iron in BLM and its relationship to the well-characterized heme systems thus continues to be an active area of research.[12]

The chemistry of ssDNA cleavage has also been extensively investigated, and work from many laboratories is consistent with the mechanism postulated in Figure 2.[8,13,14] Only 4′-hydrogen abstraction has been observed with duplex DNA and iron BLM,[15,16a] resulting in two monomeric products, base and base propenal. Identical kinetic isotope effects are observed on the formation of both products when [4′-^{2}H]dNTPs are incorporated into the DNA. Sequence-specific isotope effects can be monitored using polyacrylamide gels and specifically deuterated DNAs.[17] These observations are consistent with the formation of a common intermediate presumably containing a 4′-radical (Figure 2). The subsequent partition ratio depends on the concentration of O_2 and the sequence context. The detailed chemistry of these pathways has been reported.[8]

Recent studies from the Giese laboratory have shed additional light on pathway A (Figure 2). Using chemical methods to specifically generate a C-4′ radical in a single-stranded oligonucleotide, the first detection of the proposed peroxy radical (by electron spin resonance (ESR) spectroscopy) and hydroperoxide (by matrix-assisted laser desorption ionization−time of flight (MALDI-TOF) mass spectrometry) intermediates has been reported. Both intermediates presumably result from trapping of a 4′-nucleotide radical with O_2.[18] It is important to note, however, that these model systems which give similar and additional products to those observed with BLM and DNA result from chemistry different from that occurring in BLM-mediated DNA degradation. The products of these model reactions are consistent with rapid formation of a cation radical intermediate (Figure 3),[18] initially suggested from pulse radiolysis experiments (under

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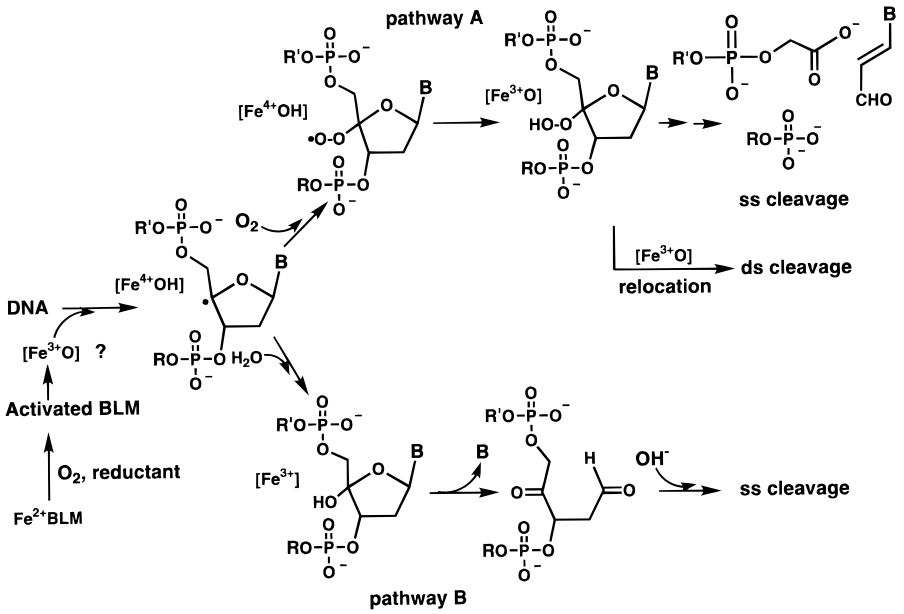

Figure 2. Proposed mechanism of FeBLM-mediated DNA cleavage (ss = single strand and ds = double strand). Pathway A requires O_2 over and above that required to form activated BLM, and pathway B requires no additional O_2 and leads to strand scission only under alkaline conditions.

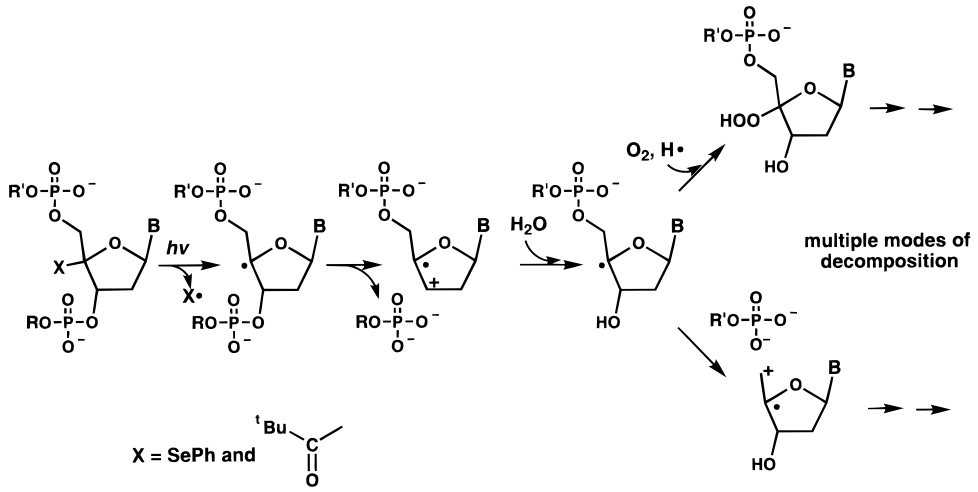

Figure 3. Proposed mechanism for products generated from a 4′-nucleotide radical intermediate produced by photolysis of 4′-substituted nucleotides.[18a,19a] The products generated are not consistent with thsoe observed in BLM-mediated DNA degradation.

anaerobic conditions) of Schulte-Frohlinde and von Sonntag.[19]

The distinction between the model work of Giese and the observed chemistry of BLM must reflect the relative rates of various reactions and the juxtapostion of a "reducible" iron center adjacent to C4′ in the case of the FeBLM system. Thus, loss of phosphate, reported by Behrens et al.[19b] to occur with a k_{obs} of $\sim 10^3\ s^{-1}$ and in model nucleotides by Giese et al.[18c] to occur with a k_{obs} of $\sim 10^5\ s^{-1}$, must be slower than the diffusion-controlled rate of reaction of O_2 with the 4′-radical or oxidation of this radical to a carbocation by the putative $Fe^{4+}OH$ species (Figure 2). It is difficult however to completely exclude the possibility that this pathway occurs as a minor process that could go undetected with available analytical methods.

This understanding of the mechanism of ssDNA cleavage has been essential for designing experiments to probe the mechanism of dsDNA cleavage. In addition, the demonstration of the importance of the 2-amino group of G in the dGpT(C) sequences experiencing cleavage suggests that the chemistry and specificity of cleavage is dependent on the minor groove environment and has stimulated much speculation on the determinants of this specificity.[20−23] Recent 2D NMR studies summarized below have now provided a framework for explaining these observations.

A Structural Model for HOO−CoBLM Bound to DNA

As summarized in Scheme 1, HOO−Fe^{3+}BLM is the last detectable intermediate before DNA degradation.

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However, the use of NMR spectroscopy to determine the structure of iron BLM is problematic for several reasons. First, Fe^{3+}BLM is paramagnetic and broadens the protons of greatest interest. Second, Fe^{3+}BLM is not very stable[10] and undergoes substantial decomposition during the time required for data acquisition. Third, the binding specificity of Fe^{3+}BLMs to DNA has not been well studied. We and others have therefore sought to use alternative metallo-BLMs for NMR analysis.[24−30] The Oppenheimer and Hecht laboratories performed the first NMR experiments in 1979 on ZnBLM and CO−Fe^{2+}BLM.[29a,b] These complexes have recently been restudied using 2D homonuclear and heteronuclear NMR spectroscopy by Akkerman et al.[30] All of the protons and carbons in these complexes have been assigned, and structures of the complexes have been proposed. In the case of both the ZnBLM and the CO−Fe^{2+}BLM complexes, the amino group of the carbamoyl moiety of mannose has been assigned as an axial ligand. This assignment is based on the proton chemical shift of H3′ of the mannose relative to the same proton in metal free BLM, the well-defined orientation of the β-hydroxyhistidine relative to the sugars in comparison with metal free BLM, and the coupling constants for the α- and β-protons of β-aminoalanine. While these data led them to favor the carbamoyl ligation over the primary amine of β-aminoalanine in the case of CO−Fe^{2+}BLM, the latter alternative could not be unambiguously ruled out.[30b] In addition, the screw sense of the isomers of these and other complexes has not been unambiguously identified. Much research is still being devoted to the characterization of a variety of the metallo-BLMs with the intent of resolving these structural issues.[12b,c,26a,d,28a] Of course, the possibility needs to be considered that perhaps these features may differ among various metallo-BLMs.

Recently the Petering and Otvos laboratories[26] and our laboratories[28] have focused on the HOO−CoBLM originally described by Chang and Meares.[31a] This compound is appealing for a number of reasons. First, it is an analog of the activated BLM (HOO−FeBLM) and, under conditions of photochemical activation,[31b] will cleave DNA with the same sequence specificity as activated FeBLM and with exclusive 4′-hydrogen abstraction.[16a,31c,33−35] Second, Co^{3+} is diamagnetic, as is the case for the ZnBLM and CO−FeBLMs, and is amenable to NMR spectroscopic analysis. Third, the ligands are exchange inert and sufficiently stable for the extended periods of time required for data acquisition by NMR methods. Xu et al. in 1994 reported the first structures for a mixture of CoBLMs: A2 green and A2 brown.[26a] The CoBLM A2 green is the hydroperoxide form (**II**), while the CoBLM A2 brown (**III**) has a slowly exchangeable H_2O (or OH^-) as an axial ligand. The successful resolution of these two species[28a] has allowed us to obtain additional NOE and dihedral angle constraints for **II**, not reported by Xu et al.[26a]

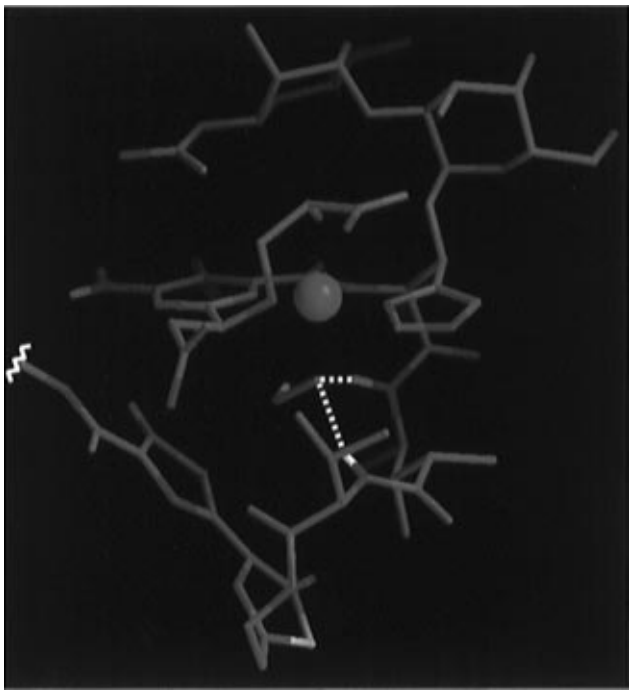

Figure 4. Solution structure of CoBLM A2 green with the primary amine of the A moiety as an axial ligand (atoms colored by element, C = green, O = red, N = blue, S = yellow, H = white). The sulfonium moiety and most of the hydrogen atoms have been removed for the sake of clarity. The dotted lines indicate the H-bonding interactions between the penultimate oxygen of the hydroperoxide axial ligand and the hydrogens of the amide moieties of T and V (Figure 1).

Detailed molecular modeling studies using these constraints from our NMR data,[28a] in addition to a variety of chemical and biophysical data summarized by Wu et al.,[28a,b] have allowed us to favor the screw sense isomer reported in Figure 4, with the primary amine of the β-aminoalanine moiety (Figure 1) as the axial ligand rather than the amino group of the carbamoyl moiety of mannose. Studies of Dabrowiak with a hydrolyzed product of BLM (pseudotetrapeptide A),[32c,37a] as well as recent crystallographic studies of

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Tan et al. on CoPMA,[36,37a] a model of CoBLM, indicate that the axial ligand can be the primary amine of the β-aminoalanine.[37b] In addition, studies of Sugiyama et al. using synthetic models in which the pyrimidine is replaced with substituted pyrimidine (PYMLs) further demonstrate the importance of the β-aminoalaninamide.[38] Very recently Boger's lab has synthesized BLM A2 without the mannose group and Hecht's group has previously prepared deglyco- and decarbamoyl-BLM A2. The DNA cleavage with Fe for all of these BLMs has been found to be almost identical to that for BLM A2 in sequence selectivity and comparable in efficiency.[29c,39c,f] These studies suggest that, at a minimum, the carbamoyl group of the mannose is not required as an axial ligand. Finally, the screw sense of the ligand organization has been established by our recent structural studies of **II** complexed to several duplex oligonucleotides.[28a,b] Only the organization of the ligands shown in Figure 4 and discussed in detail in Wu et al.[28a,b] can account for the observed NMR constraints and the cleavage chemistry.

Several remarkable features of the structure of **II** warrant highlighting. First, the bithiazole tail is folded underneath the metal binding domain on the same face as the hydroperoxide ligand (Figure 4, a refined model of one of the two structures originally proposed by Xu et al.[26a]). Second, the valeryl moiety of the peptide linker is in a well-defined conformation, which is virtually identical to its conformation when bound to the DNA. Third, modeling suggests that there are H-bonding interactions between the penultimate oxygen of the hydroperoxide axial ligand and the hydrogens of the amide moieties of T and V (Figure 4). These interactions may account for the unusually high stability of this hydroperoxide.[28a,b]

Titration studies of CoBLMs with generic DNA suggested that the DNA complex of **II** is in slow exchange with free **II** and DNA on the NMR time scale.[26c] We were encouraged by this observation and focused on the crucial choice of a defined duplex oligonucleotide with a single binding and cleavage site for **II** which could serve as the basis for developing a structural model. Our studies resulted in the selection of two oligonucleotides for investigation: d(CCAG-GCCTGG) (**1**) and d(CCAGTACTGG) (**2**) [the underlined pyrimidine indicates the site of cleavage]. Both possess a single cleavage site and a single binding site, the former determined by gel electrophoresis and the latter quantified by monitoring fluorescence quenching with Scatchard analysis, affording K_d values of $\sim 10^{-7}$ M.[28a]

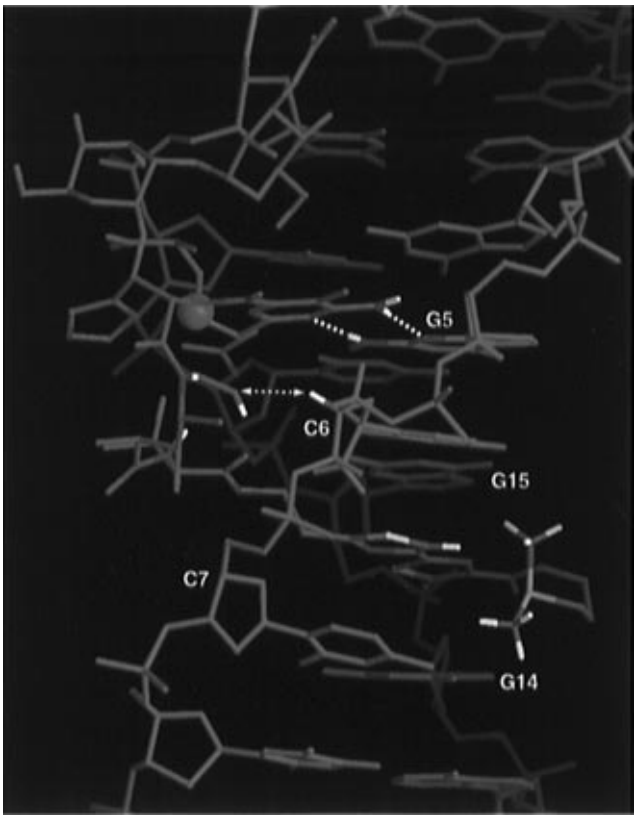

Figure 5. Structure of CoBLM A2 green (atoms colored by element, C = green, O = red, N = blue, S = yellow, H = white) bound to DNA (purple; C6−H4′ = white). The cleaved strand (C1-C2-A3-G4-G5-C6-C7-T8-G9-G10) is in the foreground, running 5′ → 3′ from the upper right to lower left corner. The dotted lines indicate the H-bond interactions between the P moiety (Figure 1) of CoBLM and the G5 of the DNA. Also indicated is the proximity of the distal oxygen of the hydroperoxide ligand to the C6−H4′ (2.5 Å).

The details of the interaction between **1** and **II** have recently been described.[28b,c] Approximately 85% of the protons associated with **II** and 95% associated with **1** have been assigned. This has resulted in the detection of 60 intermolecular NOEs, 61 intramolecular NOEs within **II**, and >200 NOEs within **1**. These constraints have been used in modeling and molecular dynamics calculations, resulting in a model of the structure shown in Figure 5. This model provides considerable insight into the three key properties of the interaction of BLMs with DNA: binding, sequence specificity, and cleavage chemistry. The bithiazole tail is a major binding determinant. It is inserted 3′ to the cleavage site from the minor groove between C6·G15 and C7·G14, indicative of a partial intercalative mode of binding. The basis for sequence specificity is defined for the first time. The N3 and the amino group of the pyrimidine of CoBLM (Figure 1) form H-bonding interactions with one of the hydrogens of the 2-amino group of G5 and the N3 of G5, respectively. Finally, the unexpected identification of the hydroperoxide proton in the complex has unambiguously defined the basis of chemical specificity. The terminal oxygen of the hydroperoxide is located 2.5 Å from the C4′H (Figure 5), a surprising result given that no constraints involving this interaction were

(36) Tan, J. D.; Hudson, S. E.; Brown, S. J.; Olmstead, M. M.; Mascharak, P. K. *J. Am. Chem. Soc.* **1992**, *114*, 3841−3853.

(37) (a) Pseudotetrapeptide A includes A, P, H, and V moieties of BLM in Figure 1; PMA is 2-[[*N*-(aminoethyl)amino]methyl]-4-[*N*-[2-(4-imidazolyl)ethyl]carbamoyl]-5-bromopyrimidine. (b) Although neither of these compounds possesses a carbamoyl ligand, these studies substantiate the feasibility of the primary amine as an axial ligand to the cobalt. In addition, to our knowledge, no crystallographic examples have been reported in which a carbamoyl group serves as an axial ligand to cobalt.

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(39) (a) Boger, D. L.; Colletti, S. L.; Honda, T.; Menezes, R. F. *J. Am. Chem. Soc.* **1994**, *116*, 5607−5618. (b) Boger, D. L.; Honda, T.; Dand, Q. *J. Am. Chem. Soc.* **1994**, *116*, 5619−5630. (c) Boger, D. L.; Honda, T.; Menezes, R. F.; Colletti, S. L. *J. Am. Chem. Soc.* **1994**, *116*, 5631−5646. (d) Boger, D. L.; Honda, T. *J. Am. Chem. Soc.* **1994**, *116*, 5647−5656. (e) Boger, D. L.; Teramoto, S.; Honda, T.; Zhou, J. *J. Am. Chem. Soc.* **1995**, *117*, 7338−7343. (f) Boger, D. L.; Teramoto, S.; Zhou, J. *J. Am. Chem. Soc.* **1995**, *117*, 7344−7356. (g) Boger, D. L.; Colletti, S. L.; Teramoto, S.; Ramsey, T. M.; Zhou, J. *Bioorg. Med. Chem.* **1995**, *3*, 1281−1295.

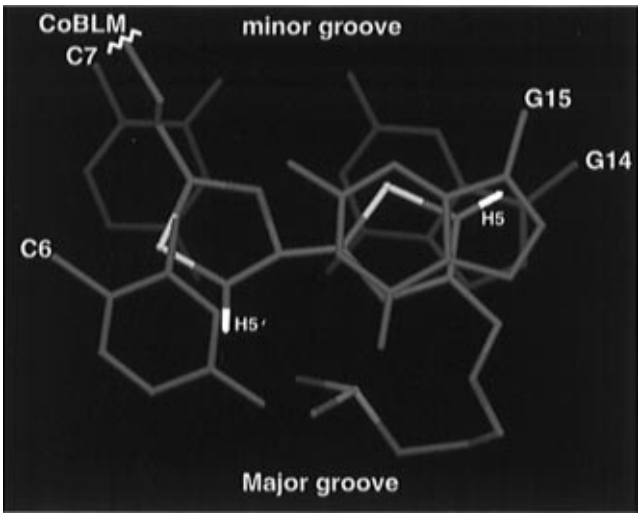

Figure 6. Binding by partial intercalation of the bithiazole tail of CoBLM A2 green to DNA. A view looking down the helical axis showing the terminal thiazolium ring stacked between the bases of G14 and G15 and the penultimate thiazolium ring partially stacked between the bases of C6 and C7.

used in the modeling. Each of these three points will be discussed below.

Examination of the binding of the bithiazole tail reveals that the terminal thiazolium ring is completely stacked between G15 and G14, while the penultimate thiazolium ring is only partially stacked between C6 and C7 (Figure 6). The H5 and H5′ protons of the thiazolium rings (Figure 1) are trans to one another. Both these protons are upfield shifted in the NMR spectrum when **II** is bound to the oligomer. We have also observed similar upfield shifts of these protons when **II** is bound to oligomer **2**. Whether this mode of binding is general remains to be established. However, the data are consistent with many of the previous studies on apoBLM (using linear dichroism, NMR, DNA unwinding measurements, fluorescence quenching under conditions of differing ionic strength, etc.), suggesting a partial intercalative mode of binding.[40−44] This binding mode also explains previous studies with phleomycin (PLM), a BLM analog in which the penultimate thiazolium ring is reduced. PLM displays a sequence selectivity and efficiency of cleavage that are similar to those of the corresponding BLM.[45,46] This result has suggested to many that intercalation cannot be the mode of binding.[42,46,47] However, molecular modeling using the structure in Figure 5 shows that PLM can be readily accommodated without significant perturbation of the structure. This type of intercalation is supported by many chemical and biochemical studies, suggesting that the bithiazole tail provides no specific interaction with DNA, just the binding energy.[7,39a,48] Finally, intercalation 3′ to the cleavage site is also consistent with diethyl pyrocarbonate and permanganate cleavage studies with CoBLM[47] and cleavage studies with FeBLM and bulged DNA.[49]

In contrast, recent studies from the Hecht lab using ZnBLM bound to an oligomer (CGCTAGCG) revealed multiple binding modes.[27] The ZnBLM clearly has a ligand coordination environment different from those of CO−FeBLM and HOO−CoBLM, and thus the observed differences are not surprising.[50] ZnBLM does not effect strand scission, and the relevance of the ZnBLM to the structure of the active metallo-BLM(s) *in vivo* thus remains to be established.

The requirement of the 2-amino group of G, 5′ to the Py cleavage site, is a feature of the sequence specificity of BLM which is generally agreed upon.[16b,20−24] However, the role of this G as a specificity determinant has remained speculative. Our studies reveal an unusual base triple (Figure 5) between the P moiety of BLM and G5 of the DNA oligomer. One of the protons of the amino group of the pyrimidine is downfield shifted (from 7.94 or 7.73 ppm in the CoBLM to 10.22 ppm in the complex) and is H bonded to the N3 of the G5. A similar downfield shifted proton is observed in the GTAC sequence (**2**), suggesting that, in this case as well, it is involved as a specificity determinant. The second key feature of the specificity is a H bond between the N3 of the pyrimidine of CoBLM and one of the 2-amino protons of G5. This result explains why replacement of G with an inosine in a GpPy sequence dramatically reduces cleavage by BLM and why cleavage at ApPy sequences is less efficient than at the GpPy sequences.[22,23,34] It is important to note that neither of these hydrogen bonds was used as a constraint in the modeling and molecular dynamics calculations.[28b]

II is postulated to be an analog of activated FeBLM. Therefore, it was hoped that a model of the structure of activated BLM would provide some insight into the mechanism of 4′-hydrogen atom abstraction (Figure 5). The structural determination was facilitated by the unexpected observation of the proton of the hydroperoxide at 8.89 ppm in H_2O. The assignment of the proton was confirmed by the recent determination of the structure of the CoBLM brown form (**III**) in which the hydroperoxide is replaced with either a H_2O or OH^- ligand. While the binding of **III** to **1** is similar to that observed for **II**,[51] the proton resonance at 8.89 ppm is absent. There are six intermolecular NOEs between the hydroperoxide proton of **II** and the DNA. These constraints in conjunction with molecular dy-

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(49) Williams, L. D.; Goldberg, I. H. *Biochemistry* **1988**, *27*, 3004−3011.
(50) Recent studies by Otvos et al.[26d] using ^{113}Cd NMR, $^{113}Cd-^{13}C$ coupling constants, and ^{13}C line width analysis reveal that CdBLM undergoes ligand reorganization between 5 and 54 °C. Temperature dependence of ^{13}C line widths is also observed with ZnBLM. In light of this work and recent crystallographic evidence of a trigonal-bipyramidal structure of a ZnBLM model compound (Kurosaki, H.; Hayashi, K.; Ishikawa, Y.; Goto, M. *Chem. Lett.* **1995**, *8*, 691−692), the ligand coordination and exchange behavior in ZnBLM need to be reinvestigated.

namics calculations as noted above have placed the terminal oxygen within 2.5 Å of the 4′-hydrogen of C6 that would be abstracted by the activated FeBLM.

Proposals for the mechanism of hydrogen abstraction by activated FeBLM are largely based on heme iron chemistry, thus favoring a heterolytic cleavage of the oxygen−oxygen bond of the hydroperoxide to generate an iron oxene or its equivalent. By analogy with the well-characterized heme peroxidases, this type of cleavage would be facilitated by general acid catalysis, i.e., proton transfer to the terminal oxygen.[52] In the case of **II** bound to **1**, however, as in the case of **II** in solution, the H-bonding is to the penultimate oxygen, the one directly bound to the cobalt. The environment of this peroxide is very sequestered with multiple H-bonding interactions, accounting for its detection and stability. The model structure is, therefore, contradistinctive to that "expected" on the basis the heme peroxidase chemistry. Unfortunately, the resolution of this model structure is not sufficiently high to warrant further speculation on the chemistry of activation and hydrogen abstraction.

Relationship of HOO−CoBLM Structure with HOO−FeBLM

A brief survey of the extensive FeBLM literature provides a number of observations to support the proposal that **II** is an excellent analog of activated FeBLM. First, the specificities of cleavage of both species are very similar, with the 2-amino group of G playing a key role in this process. It will be interesting to insert 3-deazaguanine in place of guanine in GpPy sequences to further assess similarities (or differences) between these systems, as this substitution would eliminate one of the H bonds defining the specificity. The complementary experiment, addition of a 2-amino group to an adenine (to generate 2,6-diaminopurine, DAP), has recently been reported by Bailly et al.,[23] and their results using FeBLM are completely consistent with those of our model: weak cleavage sites at ApC-(T) sequences were transformed into strong cleavage sites at the DAPpC(T) sequences. Second, the mode of binding of **II** now offers a reasonable explanation for why PLM has similar specificity and cleavage efficiency to those of FeBLM. Molecular modeling based on the structure in Figure 5 suggests that these observations do not require a minor groove binding model as proposed by many researchers.[22,53] Third, studies of several groups have shown that activated FeBLM, in the absence of DNA, can undergo self-inactivation by modification of the bithiazole tail.[54] Whether this reaction is inter- or intramolecular is not known. These data can be explained by a conformation of activated FeBLM similar to that of **II** in which the tail is folded underneath the metal binding domain and on the same face as the hydroperoxide. Fourth, **II** is a competitive inhibitor of activated FeBLM, with respect to both its ssDNA and dsDNA cleavages.[31c,33,55b] Fifth, both compounds can abstract the 4′-hydrogen atom from the deoxyribose of a Py and give the same product, a 4′-keto-1′-aldehyde with the DNA strand intact.[13b,35,56] The mechanism of this process with **II** under photochemical conditions, however, has yet to be defined. Sixth, studies with FeBLM on nucleosomes of known structure in comparison with the corresponding naked DNA revealed a drastic suppression of DNA cleavage, although specificity was identical, regardless of the positioning of the minor groove relative to the nucleosome.[57] Surprisingly, cleavage is not dictated by the accessibility of the minor groove. One interpretation of these results is steric constraints imposed by the nucleosome structure prevent conformational reorganization required for intercalation of BLM which is essential for efficient damage. Also revealed in these studies is the same extent of inhibition in ssDNA and dsDNA cleavages,[57] an observation consistent with a model in which a single molecule of BLM can effect dsDNA cleavage.[55,58]

On the basis these considerations, we believe that **II** is an excellent model for activated FeBLM. However, the CO−FeBLM has also been proposed to be an analog of the O_2−Fe(II)BLM complex, the direct precursor to HOO−FeBLM, and as summarized above is proposed to have different coordination geometry than **II**.[30b] We believe these possibilities are not mutually exclusive. If arrangements of the ligands in the CO−FeBLM complex are identical to those in Figure 4 where CO is equivalent to the HOO^-, the only point of disagreement is the assignment of the other axial ligand. As outlined above, in the case of HOO−CoBLM, we strongly favor the assignment of the axial ligand to the primary amine of β-aminoalanine. The carbamoyl ligation state, as discussed by Wu et al.,[28b] is energetically less favorable. The user-friendly synthesis of BLM with appropriate ^{15}N-labeled ligands in conjunction with a variety of physical methods is in progress to allow a resolution to this problem.

Double Strand Cleavage

In addition to catalyzing ssDNA cleavage, BLM can catalyze dsDNA cleavage. The ratio of these processes (ds:ss) has been reported by researchers in a number of laboratories and varies from 1:6 to 1:20 depending on the assay method.[4] Recent studies of Absalon et al. have observed a ratio of 1:3 in a GTAC sequence, apparently a hot spot for dsDNA cleavage.[55] The paucity of dsDNA cleavage events and the technical problems associated with investigating them have limited the available information required to understand this process.

(51) Our unpublished results indicate that **III** binds to d(CCAGGC-CTGG) by intercalation of the bithiazole tail 3′ to the cleavage site (C). Mao et al. have recently reported preliminary NMR studies with **II** and **III** and d(GGAAGCTTCC).[26e] These studies reveal a binding mode for **II** similar to what we have reported,[28b] although their assignment of the exchangeable proton at 10.22 ppm to a proton of the amino group of G5 is perhaps incorrect.[28b] However, their studies with **III** contrast with our results, and indicate that **III** is in rapid exchange on the NMR time scale with no detectable intermolecular NOEs between the bithiazole tail and the oligonucleotide.[26e] The reasons for the differences observed when **III** interacts with different oligonucleotides remain to be explained.

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5'-G-**Py**-Py-Pu-3' 5'-G-**Py**-Pu-Py-3' 5'-G-**Py**-Pu-Pu-3'
3'-C-Pu-Pu-Py-5' 3'-C-Pu-Py-Pu-5' 3'-C-Pu-Py-Py-5'

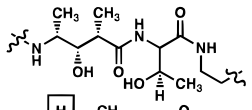
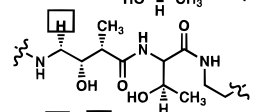
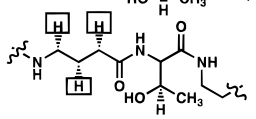
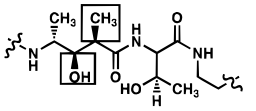
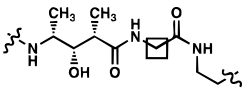
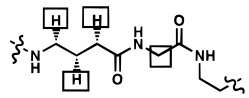
Figure 7. Sequence specificity of Ds cleavage.[58a] Bold and underlined Py denote primary and secondary cleavage sites, respectively.

The specificity of dsDNA cleavage was examined in Povirk's laboratory using nondenaturing polyacrylamide gel electrophoresis and is shown in Figure 7.[58] The examination of 23 dsDNA cleavage sites led to the proposal by Povirk that there is a primary site which is a good ssDNA cleavage site (G**Py**) and that there is a secondary site of cleavage that is determined by whether a pyrimidine or a purine is located 3′ to the Py in the primary cleavage site. DsDNA cleavage leads to blunt-ended or 5′-staggered DNA fragments (Figure 7). Absalon et al. have examined another 20 dsDNA cleavage sites using the same methods and observed cleavage sites similar to those reported by Povirk,[55c] in addition to a number of sites that had not been previously observed. Thus, a general rule of sequence specificity for dsDNA cleavage is difficult to infer in light of the relatively small amount of sequence space that has been studied for this phenomenon.

The most compelling mechanistic model for dsDNA cleavage has been proposed by Povirk and co-workers on the basis of a number of interesting experiments.[58] Many of the features of this model have been recently confirmed by using hairpin oligonucleotides designed to have a single dsDNA cleavage site, which allows quantitation of both ss and ds cleavage events.[55] The basic premise of the Povirk model is that a single molecule of BLM catalyzes both cleavage events without dissociating from the DNA and that FeBLM must be reactivated for the second cleavage event to occur. The cleavage specificity (Figure 7) indicates that this single BLM molecule must move 15−18 Å to participate in C4′-hydrogen atom abstractions on both strands.[55b] The studies of Povirk et al. and of Absalon et al. indicate that the chemistry of cleavage appears to be identical to that previously established for ssDNA cleavage. Isotope effect studies using [4′-^{2}H]Py in the dsDNA cleavage sites indicate that identical isotope effects are observed on both ssDNA and dsDNA cleavage events, suggesting that the modes of binding for both events are identical.[55b] Subsequent to hydrogen atom abstraction, the 4′-radical can lead to two types of products. However, the dsDNA cleavage process can only be observed when the O_2-dependent pathway occurs at the primary site (pathway A, Figure 2), leading to the proposal that some intermediate in this pathway is required to reactivate the FeBLM for the second cleavage event.[55b,58a] Reactivation could involve reduction of the peroxy radical concomitant with $Fe^{4+}OH$ oxidation to Fe^{5+}=O (equivalent to [Fe^{3+}O]) (Figure 2). It should be noted, however, that the events after the formation of HOO−FeBLM (intermedite **I** in Scheme 1) are unknown and there is no chemical precedent for this reactivation. This proposal, therefore, is speculative and serves as our working hypothesis.[55b,c]

The model for dsDNA cleavage predicts that the partitioning ratio between ssDNA and dsDNA cleavage depends ultimately on the relative rates of FeBLM dissociation versus reorganization to the second site where the second cleavage event can occur. Thus, every ssDNA cleavage event could lead, with some probability, to dsDNA cleavage. The structure suggests that, during this reorganization, the bithiazole tail is retained within its intercalation site and that the linker peptide (Figure 1) serves as a tether to allow for the translocation of the BLM metal binding domain from the first to the second site. The importance of the peptide linker has only recently been recognized in this process with the solution of the structure[28b] and with the studies from Boger's laboratory.[39a,c,g] In the latter case, changes in the peptide linker affect the ratio of ssDNA to dsDNA cleavage (Table 1). For example, the replacement of the threonine moiety with a glycine residue reduced the ratio of ssDNA to dsDNA cleavage by ∼3-fold (Table 1). The detailed structural analysis of these linker mutants complexed to defined DNA sequences is in progress as a complement to the cleavage studies in an effort to understand the role of the linker in the dsDNA cleavage process.

Table 1. Comparison of BLM Analogs Modified in the Linker Region: Efficiency and Ratio of Double Strand to Single Strand Cleavage[a]

	Agents	ds:ss	Efficiency
	Bleomycin A2	1:6	~3.5
Deglyco-BLM		1:12	1.0
Deglyco-		1:15	0.5
Deglyco-		1:13	0.3
Deglyco-		1:13	0.6
Deglyco-		1:29	0.2
Deglyco-		1:33	0.1
	Fe^{2+}	1:98	0.04

[a] Data taken from Boger et al. (1995).[39g] The boxed areas indicate changes in the linker region.

The basis of sequence-specific ssDNA cleavage revealed in the structure (Figure 5) has allowed us to begin consideration of the structural basis for the sequence specificity for dsDNA cleavage. Of all the dsDNA sequences that we have examined, only a single hot spot has been detected:

5′-G-**T**-A-C-3′
3′-C-A-T-G-5′

Furthermore, studies of Povirk et al.[58a] have revealed

(59) Keller, T. J.; Oppenheimer, N. J. *J. Biol. Chem.* **1987**, *262*, 15144−15150.
(60) Mirabelli, C. K.; Ting, A.; Huang, C.-H.; Mong, S.; Crooke, S. T. *Cancer Res.* **1982**, *42*, 2779−2785.

that no dsDNA cleavage has been detected in

5′-G-**C**-3′
3′-C-G-5′

sites, proposed by a number of researchers to be a likely hot spot for dsDNA cleavage.[4f,59,60] Both of these observations can easily be rationalized on the basis of the recent structure (Figure 5). A good ssDNA cleavage site (G**Py**) is preferred, if not imperative, at the first site of the dsDNA cleavage. Weak binding at the first cleavage site could lead to rapid dissociation precluding BLM's reactivation and reorganization required for dsDNA cleavage. In contrast, tighter binding involving two hydrogen bonds between the guanine in G**Py** and the pyrimidine of BLM will likely exhibit a slower dissociation rate of BLM from DNA and in turn permit sufficient time for the metal to reactivate and the metal binding region to relocate to the secondary cleavage site. As for the second cleavage site, although a good ssDNA cleavage site is not obligatory (Figure 7), the hot spot for dsDNA cleavage has a G 5′ to the second cleavage site, thus potentially offering the same full capacity for sequence recognition and binding as that observed at the primary cleavage site. Therefore, the preference of purine over pyrimidine for binding/recognition again represents an important determinant 5′ to the second cleavage site. Moreover, because the current model supports the contention that a single BLM can catalyze cleavage on both strands without dissociation, the location of thiazolium rings 3′ to the primary cleavage site places constraints on where the second cleavage can occur. This constraint thus makes it sterically impossible for BLM to cleave the second C in the

5′-G-**C**-3′
3′-C-G-5′

sequence.[61] Additional structures need to be examined to determine if the mode of binding described in Figure 5 is generic, and additional ds cleavage sites need to be elucidated quantitatively to determine if any general rules will evolve. These types of studies, in conjunction with peptide linker mutant BLMs, ought to allow, in the near future, a model for the structural basis for dsDNA cleavage to be proposed.

Summary

The new experimental information about BLM's interactions with DNA summarized above has focused on structure/function studies carried out *in vitro*. A question can be legitimately posed as to how these observations relate to the observed cytotoxicity *in vivo*.[10] While it has been demonstrated that BLM can get into the nucleus of the cell at low concentrations, when and where the metal cofactor is acquired, when and where the activation process occurs, and whether a DNA cleavage event(s) or more recently proposed RNA cleavage events[62,63] are responsible for cell-killing remain to be elucidated. Designing experiments to address these questions has been difficult due to our lack of understanding of metal homeostasis of the interplay between iron and copper metabolism and of the DNA repair. Recent studies in yeast provide an excellent model system to design experiments to address both the role of the metal[64] and the importance of ssDNA and dsDNA repair processes in BLM's cytotoxicity.[65]

The recent user-friendly total synthesis from Boger's lab,[39] coupled to the structural studies[28] and quantitative double strand cleavage assays,[55] should open a new vista for examining the structure and function of the BLMs and testing the proposed model that a single molecule of BLM effects dsDNA cleavage by reorganization involving its peptidal linker and/or the penultimate thiazolium ring. The basic principles learned from these studies could lead to the design of more effective dsDNA cleavers.

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(61) Povirk et al. have also reported dsDNA cleavage at

5′-G-**Py**-N-Pu-C-3′
3′-C-Pu-N-Py-G-5′

site.[58a] With the binding mode shown in Figure 5, a single molecule of BLM could not catalyze this cleavage.

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