## DNA Cleavage by Fe(II)·Bleomycin Conjugated to a Solid Support

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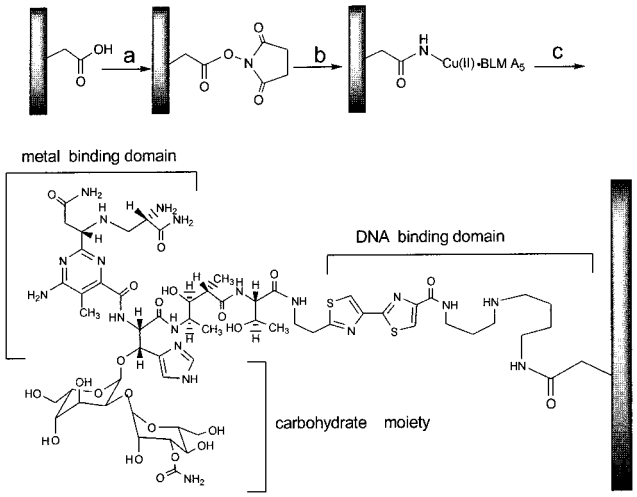
The bleomycins (BLMs) are glycopeptide-derived antitumor antibiotics.[1] BLM mediates its effects at the level of nucleic acid modification.[2] In the presence of a redox-active metal ion and oxygen, bleomycin effects sequence-selective DNA strand scission, primarily involving 5′-GC-3′ and 5′-GT-3′ sequences.[2] RNA cleavage is also highly selective and exhibits a related pattern.[2d]

BLM has three functional domains (Scheme 1). The metal binding domain is required for DNA binding, metal complexation, and oxygen binding and activation.[3] The carbohydrate moiety is involved in metal binding[4] and possibly cell permeability and selective tumor recognition.[2c,e] The C-terminus, encompassing the bithiazole moiety, as well as the cationic C-substituent, is important for DNA binding.[2]

BLM−DNA interaction has been studied for several metallobleomycins using a variety of physicochemical techniques. Good evidence exists for groove binding, intercalation and partial intercalation.[2b,c,5,6] Recently, $^1$H NMR measurements and molecular dynamics calculations have been used to study the binding of Zn(II)[5] and Co(III)[6] bleomycin congeners to specific DNA oligonucleotides. The data for all of the cobalt complexes have indicated a classically threading intercalated structure in which the bithiazole moiety is (partially) intercalated between DNA base pairs, and the C-terminal substituent has been threaded through the helix to the major groove. The zinc complexes, on the other hand, were found to involve predominantly minor groove binding with the bithiazole and C-terminal substituent in the minor groove of DNA.

All studies of BLM reported to date agree that the metal binding domain of metallobleomycins resides in the minor groove of DNA, consistent with the initiation of DNA damage in the minor groove. Therefore, if a metallobleomycin binds to DNA by threading intercalation, with the C-terminal substituent in the major groove,[6] the binding mechanism must necessarily involve the passage of some portion of BLM through the double helix. Since all modeling studies indicate that the metal binding domain is at least as large as the DNA duplex minor groove[5,6] and since separation of DNA strands in relaxed DNA is unfavorable energetically,[7] energetic considerations dictate that the much smaller C-terminal substituent must pass through the helix to effect intercalative binding. To test the consequences of increasing the size of the C-terminal substituent, we have conjugated BLM-$A_5$ to a C-terminal substituent that is far too large to permit passage through the DNA helix. Critically, we observe that the modified BLM still cleaves DNA with good efficiency and with sequence selectivity unaltered from that of free BLM-$A_5$.

**Scheme 1.** Synthetic Scheme Employed for Conjugation of BLM-$A_5$ to Solid Support[a]




[a] (a) Dioxane, *N*-hydroxysuccinimide, *N*,*N*-dicyclohexylcarbodiimide; (b) 0.1 M sodium acetate, pH 6.5, Cu(II)·BLM-$A_5$; (c) 15% aq EDTA. The functional domains of BLM are indicated.

Presently we report (i) the conjugation of BLM to a controlled pore glass (CPG) bead, creating a BLM congener having a C-terminal substituent approximately $10^5$ times larger than the remainder of the BLM molecule, (ii) the ability of the BLM conjugate to effect plasmid DNA relaxation and sequence-selective DNA cleavage, and (iii) the occurrence of DNA cleavage in a system that minimizes the opportunity for DNA strand separation to facilitate intercalation. The BLM conjugate cleaves DNA with the same sequence selectivity as free BLM and with only moderately decreased efficiency.

Cu(II)·BLM-$A_5$ was attached through the C-terminal spermidine moiety to a CPG bead functionalized with a glycolic acid linker (Scheme 1).[8] Cleavage of supercoiled pUC18 DNA was observed at a concentration as low as 0.2 $\mu$M Fe(II)·BLM-$A_5$−CPG (Figure 1).[9] At 1.0 $\mu$M concentration, Fe(II)·BLM-$A_5$−CPG afforded predominantly nicked (form II) DNA. Upon comparison with free Fe(II)·BLM-$A_5$, Fe(II)·BLM-$A_5$−CPG was found to be severalfold less potent as a DNA cleaving agent (Figure 1, cf lanes 5 and 9 and lanes 3 and 8).[10]

(1) *Bleomycin Chemotherapy;* Sikic, B. I., Rozencweig, M., Carter, S. K., Eds.; Academic Press: Orlando, FL, 1985.

(2) (a) Stubbe, J.; Kozarich, J. W. *Chem. Rev.* **1987**, *87*, 1107. (b) Natrajan, A.; Hecht, S. M. In *Molecular Aspects of Anticancer Drug-DNA Interactions*; Neidle, S., Waring, M. J., Eds.; Macmillan: London, 1980; pp 197−242. (c) Kane, S. A.; Hecht, S. M. *Prog. Nucleic Acids Res. Mol. Biol.* **1994**, *49*, 313. (d) Hecht, S. M. *Bioconjugate Chem.* **1994**, *5*, 513. (e) Hecht, S. M. In *Cancer Chemotherapeutic Agents*; Foye, W. O., Ed.; American Chemical Society, Washington, 1995; pp 369−388.

(3) (a) Sugiyama, H.; Kilkuskie, R. E.; Chang, L.-H.; Ma, L.-T.; Hecht, S. M.; van der Marel, G. A.; van Boom, J. H. *J. Am. Chem. Soc.* **1986**, *108*, 3852. (b) Carter, B. J.; Murty, V. S.; Reddy, K. S.; Wang, S.-N.; Hecht, S. M. *J. Biol. Chem.* **1990**, *265*, 4193. (c) Guajardo, R. J.; Hudson, S. E.; Brown, S. J.; Mascharak, P. K. *J. Am. Chem. Soc.* **1993**, *115*, 7971.

(4) (a) Oppenheimer, N. J.; Rodriguez, L. O.; Hecht, S. M. *Proc. Natl. Acad. Sci. U.S.A.* **1979**, *76*, 5616. (b) Akkerman, M. A. J.; Neijman, E. W. J. F.; Wijmenga, S. S.; Hilbers, C. W.; Bermel, W. J. *J. Am. Chem. Soc.* **1990**, *112*, 7462.

(5) See (a) Manderville, R. A.; Ellena, J. F.; Hecht, S. M. *J. Am. Chem. Soc.* **1995**, *117*, 7891. (b) Sucheck, S. J.; Ellena, J. F.; Hecht, S. M. *J. Am. Chem. Soc.* **1998**, *120*, 7450 and references therein.

(6) (a) Wu, W.; Vanderwall, D. E.; Stubbe, J.; Kozarich, J. W.; Turner, C. J. *J. Am. Chem. Soc.* **1994**, *116*, 10843. (b) Wu, W.; Vanderwall, D. E.; Liu, S. M.; Tang, X.-J.; Turner, C. J.; Kozarich, J. W.; Stubbe, J. *J. Am. Chem. Soc.* **1996**, *118*, 1281. (c) Cortes, J. C.; Sugiyama, H.; Ikudome, K.; Saito, I.; Wang, A. H.-J. *Biochemistry* **1997**, *36*, 9995.

(7) (a) Guéron, M.; Kochoyan, M.; Leroy, J.-L. *Nature* **1987**, *328*, 89. (b) Lilley, D. M. J. In *Bioorganic Chemistry: Nucleic Acids*; Hecht, S. M., Ed.; Oxford: New York, 1996; p 188.

(8) The CPG was activated as the *N*-hydroxysuccinimide ester and treated with Cu(II)·BLM-$A_5$ in 0.1 M Na acetate, pH 6.5, and then demetalated (15% EDTA). The extent of conjugation was determined by diminution of the supernatant absorption at 292 nm due to the bithiazole moiety ($\epsilon_{292}$ 14 500 $M^{-1}$ $cm^{-1}$).[5a] Attachment via the BLM C-substituent was verified by repeating the experiment with Cu(II)·BLM-$A_2$,[2] which afforded no conjugated product.

(9) The concentration of Fe(II)·BLM-$A_5$ employed as a CPG conjugate is expressed as the concentration that would have been present in solution had there been no conjugation to the glass bead. The beads employed for this experiment ranged in size from 50 to 100 $\mu$m ($5-10 \times 10^5$ Å) and contained approximately $4.3 \times 10^3$ fmol of BLM-$A_5$ per bead. The average distance between BLMs was about 20 Å, which should be sufficient to permit Fe·BLM activation via bimolecular collision.[2]

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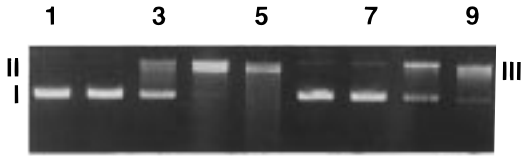


**Figure 1.** Cleavage of supercoiled pUC18 DNA by Fe(II)·BLM-$A_5$−CPG. Ten-$\mu$L reaction mixtures contained 200 ng (0.11 pmol) of plasmid DNA in 6.7 mM Na cacodylate, pH 7, at 0 °C. After 30 min, the reaction mixtures were analyzed on a 1.2% agarose gel. Lane 1, DNA alone; lane 2, 2.2 $\mu$M $Fe^{2+}$; lane 3, 0.1 $\mu$M Fe(II)· BLM-$A_5$; lane 4, 0.4 $\mu$M Fe(II)·BLM-$A_5$; lane 5, 0.8 $\mu$M Fe(II)·BLM-$A_5$; lane 6, 0.2 $\mu$M Fe(II)·BLM-$A_5$−CPG; lane 7, 0.4 $\mu$M Fe(II)·BLM-$A_5$−CPG; lane 8, 1.0 $\mu$M Fe(II)·BLM-$A_5$−CPG; lane 9, 2.2 $\mu$M Fe(II)·BLM-$A_5$−CPG.

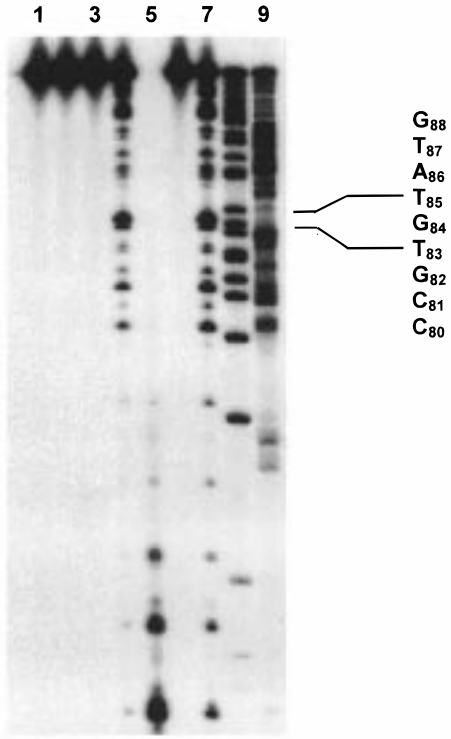


**Figure 2.** Cleavage of linear DNA duplex by tethered Fe(II)·BLM-$A_5$. A 5′-$^{32}$P end-labeled 158-bp DNA restriction fragment was incubated with 10 $\mu$M tethered Fe(II)·BLM-$A_5$ at 4 °C for 15 min and then analyzed on a 14% denaturing polyacrylamide gel. Lane 1, DNA alone; lane 2, 10 $\mu$M $Fe^{2+}$; lane 3, 10 $\mu$M BLM-$A_5$; lane 4, 1 $\mu$M Fe(II)·BLM-$A_5$; lane 5, 10 $\mu$M Fe(II)·BLM-$A_5$; lane 6, 10 $\mu$M BLM-$A_5$−CPG; lane 7, 10 $\mu$M Fe(II)·BLM-$A_5$−CPG; lane 8, G lane; lane 9, C lane.

Fe(II)·BLM-$A_5$−CPG also cleaved a linear duplex at the same sites as free Fe(II)·BLM-$A_5$ (Figure 2). Chemical sequence analysis indicated that the strongest bands occurred at 5′-$G_{82}T_{83}$-3′ and 5′-$G_{84}T_{85}$-3′, the same as those for free Fe(II)·BLM-$A_5$. The conjugated Fe(II)·BLM-$A_5$ was about 5-fold less efficient than free Fe(II)·BLM-$A_5$.[10,11]

To minimize the chances that the observed cleavage could have involved extensive denaturation of the DNA substrate to form single-stranded DNA, which then rehydribized around the conjugated BLM-$A_5$, the sequence-selective DNA cleavage studies were repeated using relaxed pBR322 DNA as a substrate. Following Fe(II)·BLM-$A_5$−CPG-mediated cleavage to afford an average of less than one nick per plasmid, the DNA was treated with restriction endonucleases *Hin*dIII and *Eco*RV and the resulting 158-bp DNA duplex was 5′-$^{32}$P end-labeled and used for sequence analysis in direct comparison with the same restriction fragment that had been excised from the plasmid prior to treatment with Fe(II)·BLM-$A_5$−CPG. Cleavage of the relaxed plasmid within the fragment analyzed occurred at the major site of damage apparent in the excised duplex. Since the relaxed plasmid DNA contained neither ends nor negative supercoiling to facilitate strand separation,[7] it seems implausible that the observed cleavage by Fe(II)·BLM-$A_5$−CPG could involve a threading intercalative mechanism.

Studies that support an intercalative mechanism of DNA binding by BLM include measurements of DNA unwinding[12] and helix elongation.[13] Recent NMR studies of Co(III)·BLM congeners[6] also provide convincing evidence that this metalloBLM binds to DNA oligonucleotides by intercalation. In comparison, the present results demonstrate that attachment of BLM to a solid support in a fashion that must preclude threading intercalation has no effect on the sequence selectivity of DNA cleavage by Fe(II)·BLM-$A_5$ and only a modest effect on cleavage efficiency.[10] In a mechanistically related study, we have shown that analogues of deglycoBLM-$A_2$ in which the bithiazole moiety contained one or two chlorine atoms gave normal sequence-selective cleavage of DNA in the presence of $Fe^{2+}$ but gave photoinduced damage (involving C−Cl bond homolysis) that clearly indicated a minor groove orientation of the bithiazole moiety.[14] We have also shown that deglycoBLM analogues lacking one of the two thiazoles failed to give sequence-selective DNA cleavage,[15] suggesting that the conjugated BLM must utilize its bithiazole moiety to effect selective DNA cleavage. The accumulated data argue strongly that, while intercalation may increase the binding affinity of BLM for DNA, threading intercalation is not required for sequence selective DNA cleavage. The present results are thus in agreement with previous findings for Zn(II)·BLM[5] and phleomycin,[16] neither of which would seem to bind to DNA by intercalation.

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(10) Although the BLM conjugate may actually be intrinsically less active than free BLM in cleaving DNA, part of the observed diminution of DNA cleavage may well be due to the localization of BLM to the surface of the glass beads (whose concentration in the reaction mixture was about $10^{12}$-fold lower than that of the conjugated BLM-$A_5$). Control experiments established that cleavage was not due to free BLM released from the CPG.

(11) Fe(II)·BLM-$A_5$ conjugated to CPG through a longer linker, or to a Sepharose resin, gave similar results. Green Co(III)·BLM-$A_5$−CPG was also prepared and gave results quite similar to those for Fe(II)·BLM-$A_5$ following photoinduced DNA cleavage.

(12) Levy, M. J.; Hecht, S. M. *Biochemistry* **1988**, *27*, 2647.
(13) Povirk, L. F.; Hogan, M.; Dattagupta, N. *Biochemistry* **1979**, *18*, 96.
(14) Zuber, G.; Quada, J. C., Jr.; Hecht, S. M. *J. Am. Chem. Soc.* **1998**, *120*, 9368.
(15) Hamamichi, N.; Natrajan, A.; Hecht, S. M. *J. Am. Chem. Soc.* **1992**, *114*, 6278.
(16) (a) Povirk, L. F.; Hogan, M.; Dattagupta, N.; Buechner, M. *Biochemistry* **1981**, *20*, 665. (b) Kross, J.; Henner, W. D.; Hecht, S. M.; Haseltine, W. A. *Biochemistry* **1982**, *21*, 4310.