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Identification and Cleavage Site Analysis of DNA Sequences Bound Strongly by Bleomycin

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Abstract: A hairpin DNA library containing an 8-base pair sequence-randomized region was employed in a SELEX-type procedure to identify DNAs that bound strongly to bleomycin A_5, the latter of which was immobilized on a solid support. Ten hairpin DNAs that bound BLM A_5 strongly were identified and sequenced, and used to characterize DNA binding by the antitumor antibiotic. While all 10 selected hairpin DNAs bound to BLM strongly, they did exhibit a range of binding specificities. Further, while the binding specificity was generally the greatest for hairpin DNAs that contained at least one of the sequences (5′-GC-3′ and 5′-GT-3′) cleaved most frequently by Fe(II)·bleomycin, the hairpin DNA exhibiting the poorest binding specificity also contained a 5′-GT-3′ site. Four of the hairpin DNA substrates were 5′-^{32}P end-labeled and used to assess the preferred sites of cleavage by Fe(II)·BLM. The substrate DNAs included two lacking any 5′-GT-3′ or 5′-GC-3′ site; these were cleaved at 5′-AA-3′ and (more strongly) at 5′-AT-3′ and 5′-GA-3′ sequences. For two hairpin DNAs containing 5′-GT-3′ or 5′-GC-3′ sequences, cleavage was observed at these sequences as well, but the three aforementioned sequences were also cleaved efficiently. For hairpin DNA **3**, which was bound the least well of the 10 DNAs studied, a 5′-TA-3′ site was also cleaved efficiently. Thus, the pattern and intensities of cleavage of the four DNAs studied were not entirely consistent with the preferred pattern of DNA cleavage reported for Fe(II)·BLM in numerous published studies that have employed arbitrarily chosen DNA substrates. Also studied were the chemistry of DNA cleavage for one of the hairpin DNAs, and competition experiments in which the diminution of cleavage was measured following admixture of a molar excess of a smaller hairpin DNA shown to be an exceptionally good substrate for cleavage by Fe(II)·BLM. In the aggregate, the data indicate that the relationship between DNA binding and degradation by Fe·BLM, as well as the chemistry leading to DNA degradation, are more complex than suggested by earlier studies that employed only DNA degradation product analysis as an end point.

Introduction

The bleomycins (BLMs) are structurally related glycopeptide-derived antitumor antibiotics originally isolated from cultures of *Streptomyces verticillus* as copper chelates by Umezawa and co-workers.[1] The clinically administered mixture of BLMs, blenoxane, comprised primarily of BLM A_2 and BLM B_2, has been found to exhibit activity against a number of cancers, including non-Hodgkin's lymphomas, squamous cell carcinomas, and testicular tumors.[2] The therapeutic efficacy of the BLMs is believed to derive from their ability to bind and oxidatively cleave cellular DNA,[3] and possibly RNA,[3c,4] in the presence of O_2 and certain metal ion cofactors.[3−5] DNA cleavage by the BLMs has been studied extensively and shown to be sequence selective, occurring primarily at a subset of all 5′-GT-3′ and 5′-GC-3′ sequences.[6] DNA cleavage involves binding of an activated metallobleomycin to DNA,[7] followed by abstraction of a sugar H atom from C-4′ of the deoxyribose

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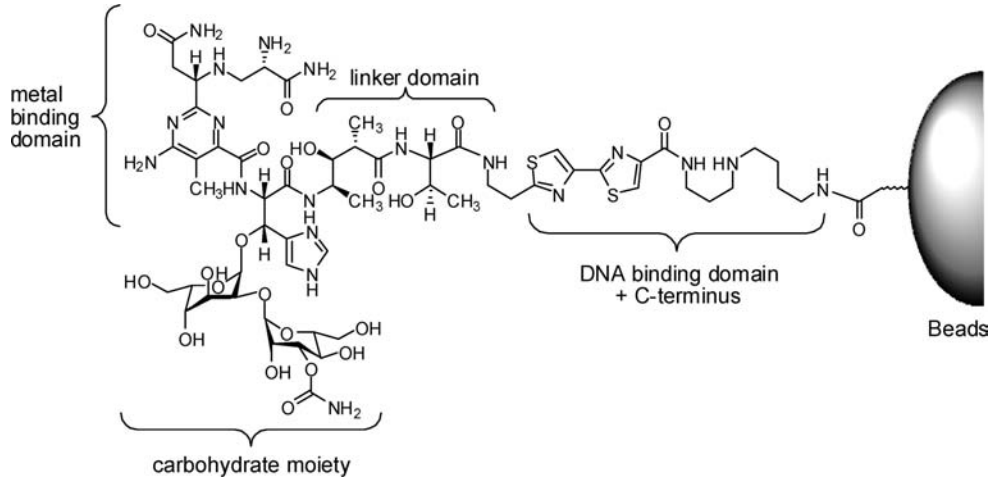

Figure 1. Structure of BLM A_5 attached through the C-terminal substituent to Sepharose 4B beads. The structural domains of bleomycin are indicated.

moiety in the rate-limiting step.[8] The ability of BLM to effect DNA degradation is strongly dependent on its molecular structure, which is organized into discrete structural domains, including a DNA binding domain, metal binding domain, linker domain, carbohydrate moiety, and C-terminal substituent (Figure 1). The metal binding domain of BLM constitutes the primary determinant of the sequence selectivity of DNA cleavage by BLMs.[9] ^{1}H NMR measurements and molecular dynamics studies indicate that the metal binding domain of metallobleomycins resides in the minor groove of DNA.[10] Recently, Goodwin and co-workers reported an X-ray crystallographic study in which they showed that the binding of BLM to DNA involves direct hydrogen-bonding interactions of the metal binding domain and disaccharide through minor groove binding.[11] While there is now compelling evidence that the metal binding domain and carbohydrate moiety of bleomycin reside in the minor groove of DNA,[10,11] and significant evidence that multiple modes of DNA binding are possible,[11,12] little is known about the actual binding selectivity of BLM or the relationship between DNA binding and cleavage sites. For a drug that is used clinically at a rather low dose ($\sim$5 μmol), it seems logical to think that DNA binding selectivity will be an important determinant of the sites that are accessible for cleavage in a clinical setting.

Recently, we described a novel strategy to identify the DNA motifs that are bound strongly by BLM (Scheme 1) and to analyze the specificity of DNA binding.[13] In the present study, we have extended this strategy to identify and compare 10 hairpin DNAs that bind BLM strongly, permitting an analysis of the structural elements conducive to DNA binding by this antitumor agent. The sequence selectivity of cleavage of four of the 10 DNAs has also been determined, permitting the first direct comparison of DNA binding and cleavage by BLM.

Results

Identification of DNAs That Bind BLM Strongly. The strategy used for the isolation of DNAs that bind BLM strongly is summarized in Scheme 1. A hairpin DNA library was created containing 8-base pair (bp) random sequences in the middle of the hairpin. Briefly, a single-strand DNA template including 8-nucleotide (nt) random sequences in the center (5′-TTTAAT-TAATXXXXXXXXAAAAAATTTTAACCCCTTAA AAT-3′, X = randomized nucleotides) was synthesized chemically and was self-annealed to form a hairpin at A_{24}-T_{41}. Treatment with the Klenow fragment of DNA polymerase in the presence of all four nucleoside 5′-triphosphates then resulted in incorporation of an additional 23 nucleotides in a template-dependent fashion, affording a 64-nt hairpin DNA library (Scheme 1). The hairpin DNA library was then incubated with resin-bound BLM A_5 (Figure 1) in 20 mM Tris-HCl buffer, pH 7.4, for 20 min. After the beads were recovered and washed with buffer, the strongly bound hairpin DNAs were removed by washing with 1 M NaCl and desalted by Microcon YM10 filtration. Digestion with restriction endonucleases *Ase*I and *Apo*I afforded a mixture of DNA duplexes with sticky ends, which were inserted into the corresponding site of predigested plasmid pT7 Blue. Following the recombinant transformation into competent cells of *Escherichia coli* DH5α, a number of colonies containing inserts from the hairpin DNAs were recovered. After sequencing of the region of the DNA encompassing the insert, 10 of the selected hairpin DNAs (**1**−**10**) whose sequences had been fully defined were synthesized chemically for biochemical characterization (Table 1). Six hairpin DNAs (**2**, **5**, **7**, **8**, **9**, and **10**) contained

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Scheme 1

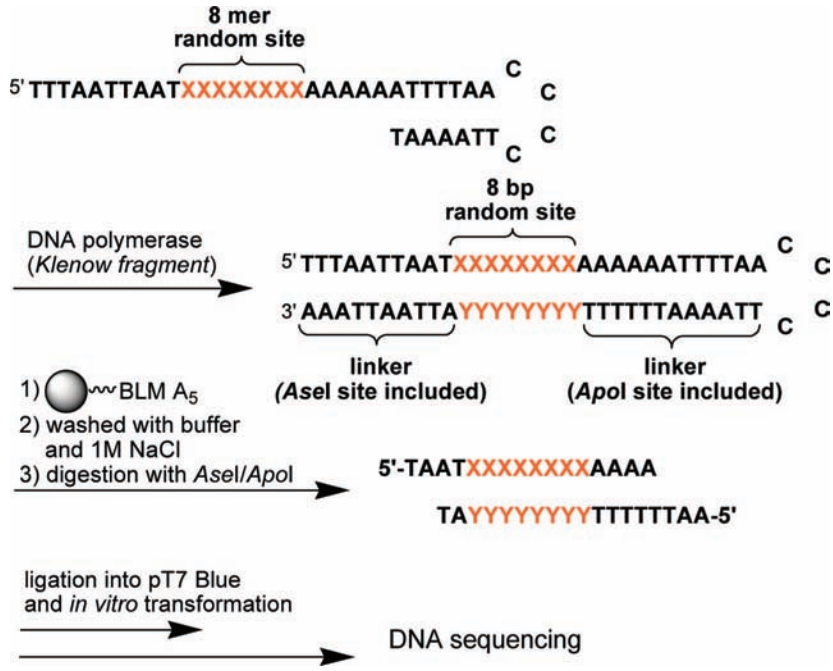

Table 1. Specific BLM Binding Sequences in 64-nt Hairpin DNA Isolated from Resin-Linked BLM A_5

5' TTTAATTAATXXXXXXXXAAAAAATTTTAA
3' AAATTAATTAYYYYYYYYTTTTTTAAAATT

1	5' AGATCATG 3' TCTAGTAC	**6**	5' CTACTAAA 3' GATGATTT
2	5'CGTGACGC 3'GCACTGCG	**7**	5'TACGCGCA 3'ATGCGCGT
3	5' TAAGTGGG 3' ATTCACCC	**8**	5' GGGTACCT 3' CCCATGGA
4	5' GAGAGGAT 3' CTCTCCTA	**9**	5' CGTTGTTA 3' GCAACAAT
5	5' ACAGAATA 3' TGTCTTAT	**10**	5' CGCCATTG 3' GCGGTAAC

Table 2. Distribution of Individual Dinucleotides within Nucleotide Regions 11−18 and 47−54 of Hairpin DNAs **1**−**10**

Dinucleotide Sequences	Hairpin DNAs									
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
AA			+		+	++			++	+
AC		++	+		+	+	+	++	++	
AG	+		+	++	+	++		+		
AT	++++			++	++					++
CA	++	+	+		+		+		+	++
CC			++	+				+++		+
CG		++++					++++		++	++
CT	+		+	++	+	++		+		
GA	++	+		+++	+					
GC		++					++++			++
GG			++	+				+++		+
GT		++	+		+	+	+	++	++	
TA			++		++	++++	++	++	++	
TC	++	+		+++	+					
TG	++	+	+		+		+		+	++
TT			+		+	++			++	+

two to five 5′-GC-3′ or 5′-GT-3′ sequences, although only hairpin DNAs **2** and **7** had both types of sequences (Tables 1 and 2). Hairpin DNAs **3** and **6** had a single 5′-GT-3′ sequence. Interestingly, two of the hairpin DNAs that were bound strongly by BLM A_5 (**1** and **4**) had neither a 5′-GC-3′ nor a 5′-GT-3′ sequence.

Fluorescence Spectra of Inhibition Assay by Hairpin DNA with Specific BLM Binding Sequence. To evaluate the BLM binding characteristics of the identified hairpin DNAs, a 16-nt hairpin DNA containing 4-aminobenzo[*g*]quinazolin-2-one 2′-deoxyribose at position C_{15}[14] (Figure 2) was used to assess the specificity of BLM A_5 binding. As described previously,[13] this was done by admixture of equimolar concentrations of Fe(II)·BLM, the profluorescent hairpin DNA-C_{f15},[14] and the hairpin DNA whose specific DNA affinity was to be determined. The profluorescent hairpin DNA-C_{f15} releases a strongly fluorescent degradation product when cleaved oxidatively by Fe(II)·BLM (Figure 3). The ability of a competitor DNA to bind the available Fe(II)·BLM tightly can be quantified as the reduction in fluorescence intensity resulting from degradation of hairpin DNA-C_{f15}. This is illustrated in Figure 3 for hairpin DNA **7**. While the initially synthesized hairpin DNA library diminished the degradation of hairpin DNA-C_{f15} only minimally (cf. green and red emission curves), hairpin DNA **7** reduced the fluorescence emission by 97% (blue curve), effectively reducing fluorescence emission close to the level observed in the absence of Fe^{2+} (black curve).

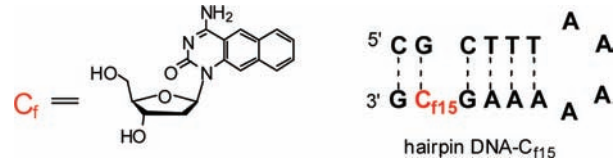

Figure 2. Chemical structure of a 16-nt hairpin DNA-C_{f15} having the 2′-deoxyriboside of 4-aminobenzo[*g*]quinazoline-2-one (C_f) at position 15.

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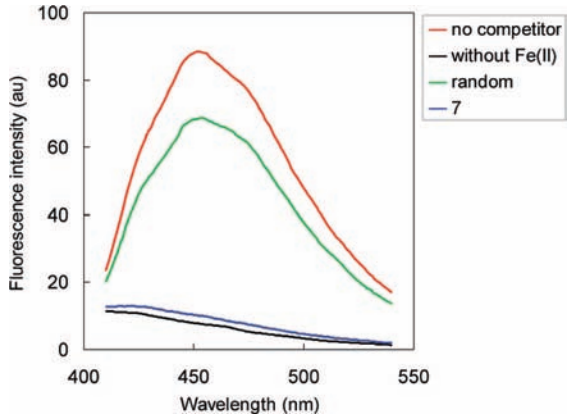

Figure 3. Fluorescence emission spectra resulting from treatment of 16-nt hairpin DNA-C_{f15} with Fe(II)·BLM in the presence or absence of 64-nt hairpin DNAs. Reaction mixture contained 0.72 μM Fe(II)·BLM, 0.72 μM hairpin DNA-C_{f15}, and 0.72 μM hairpin DNA (random or **7**) in 10 mM cacodylate buffer solution, pH 7.0, with 100 mM NaCl. The emission spectra were obtained following excitation at 310 nm at 25 °C. The emission spectrum of 16-nt hairpin DNA-C_{f15} treated with BLM A_5 in the absence of 64-nt hairpin DNA under the same conditions is also shown.

Table 3. Inhibition of Fluorescence Enhancement by Selected Hairpin DNAs[a]

hairpin DNA	binding specificity (%)	hairpin DNA	binding specificity (%)
1	82	**6**	81
2	97	**7**	97
3	76	**8**	92
4	79	**9**	89
5	90	**10**	86

[a] The binding specificity (%) was calculated as the decrease in fluorescence intensity at maximum emission wavelength (455 nm) from no competitor (0%) through the reaction mixture without Fe^{2+} (100%).

The remaining nine hairpin DNAs that had been isolated as outlined in Scheme 1 were also tested for their ability to diminish the Fe(II)·BLM-mediated degradation of profluorescent hairpin DNA-C_{f15}. As summarized in Table 3, all of the selected hairpin DNAs were quite effective in suppressing the degradation of hairpin DNA-C_{f15}, with binding specificities ranging from 76% to 97%. Hairpin DNAs **2** and **7** were the most effective in suppressing the release of the fluorescent degradation product (97% inhibition), while hairpin DNAs **3** and **4** had the lowest binding specificities (76% and 79%, respectively).

Sequence-Selective Cleavage of Four of the Selected Hairpin DNAs by Fe(II)·BLM A_5. The successful identification of DNAs bound strongly by BLM A_5 permitted the analysis of the relationship between the high-affinity DNA binding sites and cleavage efficiency and selectivity of these DNAs. Hairpin DNAs **1**−**4**, having binding specificities of 82%, 97%, 76%, and 79%, respectively (Table 3), were synthesized chemically; all of them were self-annealed to form hairpin DNAs and then subjected to cleavage by Fe(II)·BLM A_5. All four of the DNAs were found to be very good substrates for Fe(II)·BLM A_5-mediated degradation as judged by polyacrylamide gel analysis (Figures 4−7). For hairpin DNA **1** (Figure 4), there were at least six strong sites of cleavage, including A_9, T_{10}, A_{13}, T_{14}, T_{17}, and A_{19} which were quite prominent in the presence of 20 μM Fe(II)·BLM A_5. Interestingly, three of these sites (A_9, T_{10}, and A_{19}) were outside the 8-nt region randomized during library construction, and only two (5′-GA_{13}-3′ and 5′-GA_{19}-3′) involved a sequence that appears in arbitrarily selected DNA sequences as a frequent cleavage site.[6] DNA hairpin **2** contains 5′-GT_{13}-3′ and 5′-GC_{18}-3′ sites, and these were the predominant sites of

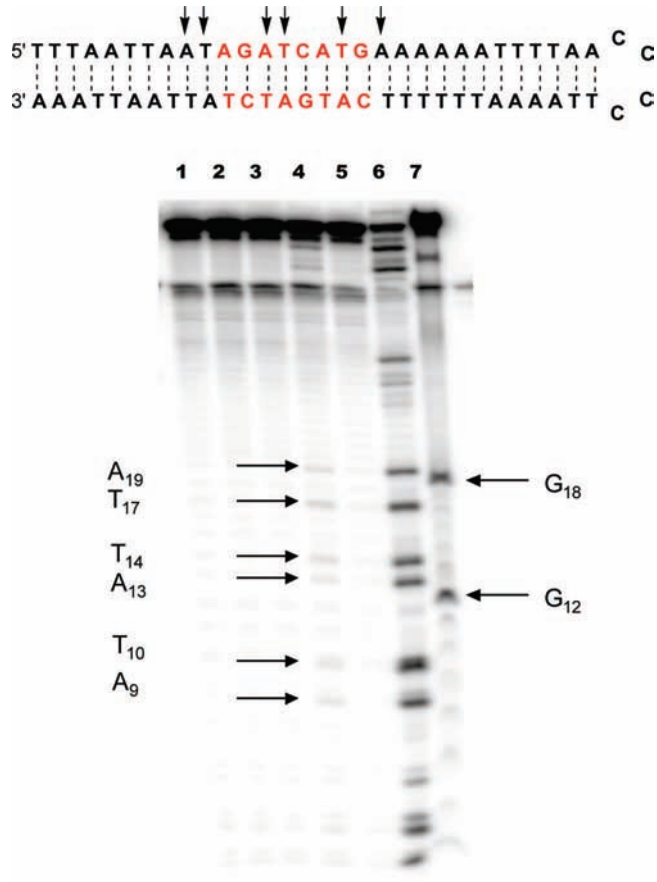

Figure 4. Sequence-selective cleavage of [5′-^{32}P]-end-labeled 64-nt hairpin DNA **1** by BLM A_5. Lane 1, radiolabeled **1** alone; lane 2, 20 μM Fe^{2+}; lane 3, 5 μM BLM A_5; lane 4, 5 μM Fe(II)·BLM A_5; lane 5, 20 μM BLM A_5; lane 6, 20 μM Fe(II)·BLM A_5; lane 7, G-lane.

cleavage in this DNA (Figure 5). Weaker cleavage was also observed at 5′-GA_{15}-3′, and at two sites (5′-AA_9-3′ and 5′-AT_{10}-3′) outside of the region that had been randomized in the initial library. It may be noted that cleavage at T_{13} and C_{18} was readily apparent at 5 μM Fe(II)·$BLMA_5$, as well as at the higher concentration (20 μM).

The cleavage of hairpin DNAs **3** (Figure 6) and **4** (Figure 7) was readily apparent, but only at 20 μM Fe(II)·BLM A_5 concentrations. For DNA **3**, six sites of cleavage were observed: A_8, A_9, T_{10}, A_{12}, T_{15}, and A_{19}. It is interesting that four of these six sites (A_8, A_9, T_{10}, and A_{19}) were outside of the original 8-nt region randomized in the initial library and that the single 5′-GT_{15}-3′ site was cleaved with an efficiency that was unremarkable relative to the other five cleavage sites. It may be noted, however, that for a sequence having multiple cleavage sites, secondary cleavage events may plausibly have biased the apparent efficiency of cleavage at T_{15}. For hairpin DNA **4**, treatment with 20 μM Fe(II)·BLM A_5 resulted in cleavage at six sites (A_9, T_{10}, A_{12}, A_{14}, A_{17}, and T_{18}), two of which were outside of the initially randomized 8-bp region. All three 5′-GA-3′ sequences in the region of nucleotides 11−18 were cleaved, and one (5′-GA_{12}-3′) was cleaved the most efficiently of the six cleaved sites in this hairpin DNA. It is interesting that there were 5′-AA-3′ and 5′-AT-3′ cleavage sites in all four of the DNAs studied, as well as two 5′-TA-3′ cleavage sites in hairpin DNA **3** (Table 4).

BLM-mediated DNA degradation involves two pathways, and the availability of oxygen influences the ratio of degradation

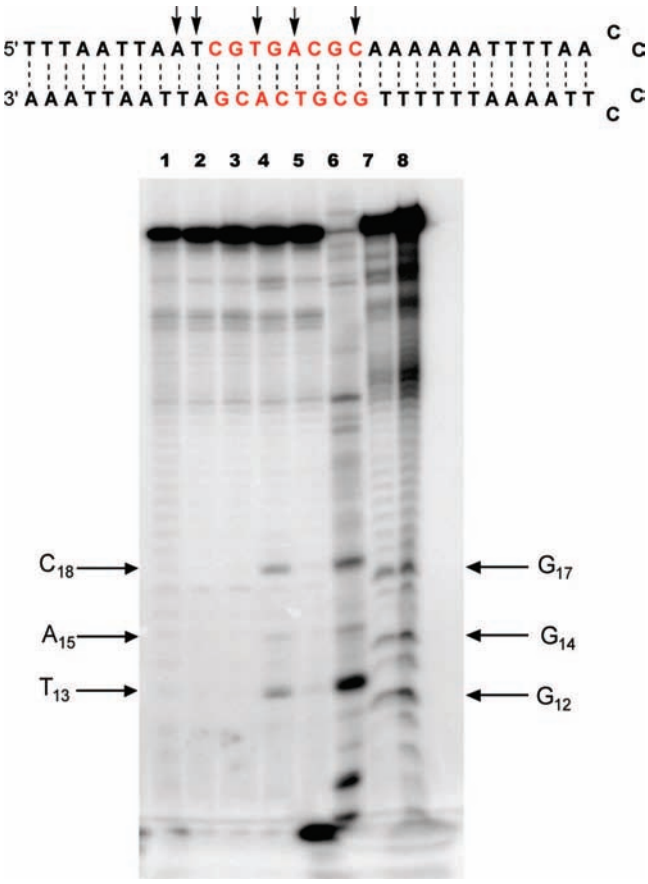

Figure 5. Sequence-selective cleavage of [5′-^{32}P]-end-labeled 64-nt hairpin DNA **2** by BLM A_5. Lane 1, radiolabeled **2** alone; lane 2, 20 μM Fe^{2+}; lane 3, 5 μM BLM A_5; lane 4, 5 μM Fe(II)·BLM A_5; lane 5, 20 μM BLM A_5; lane 6, 20 μM Fe(II)·BLM A_5; lane 7, G-lane; lane 8, A + G-lane.

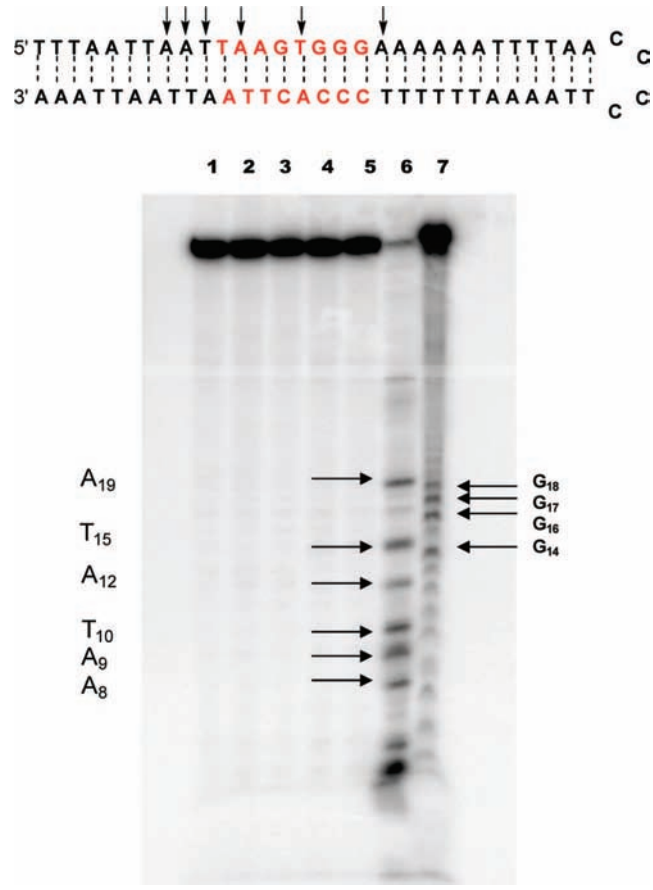

Figure 6. Sequence-selective cleavage of [5′-^{32}P]-end-labeled 64-nt hairpin DNA **3** by BLM A_5. Lane 1, radiolabeled **3** alone; lane 2, 20 μM Fe^{2+}; lane 3, 5 μM BLM A_5; lane 4, 5 μM Fe(II)·BLM A_5; lane 5, 20 μM BLM A_5; lane 6, 20 μM Fe(II)·BLM A_5; lane 7, G-lane.

using these two pathways.[3] The DNA degradation pathway that becomes prominent at low oxygen tension leads to the formation of an alkali-labile lesion.[15] Treatment of 5′-^{32}P end-labeled hairpin DNA **4** with 0.2 M *n*-butylamine following treatment with 20 μM Fe(II)·BLM produced additional products, as observed in lane 12 in Figure 7. Three additional cleavage bands were found (at G_{11}, G_{13}, and G_{15}), and the six bands observed initially all migrated more slowly following *n*-butylamine treatment (cf. lanes 11 and 12), with some of the bands appearing as double bands. In addition, new products were observed at the 5′-end of the gel in lane 12 of Figure 7. This is characteristic of the alkali-labile lesions and phosphoglycolate termini characterized previously.[3,15]

To explore the relationship between DNA binding and cleavage, competition experiments were carried out using radiolabeled hairpin DNAs **1** and **2** as substrates in the presence of an unlabeled 16-nt hairpin DNA having the same sequence as the hairpin DNA shown in Figure 2. As expected, the cleavage of hairpin DNAs **1** and **2** was significantly reduced in the presence of the 16-nt hairpin DNA, and in a concentration-dependent fashion (Figure 8). The relative efficiencies of cleavage of **1** and **2** by Fe(II)·BLM were calculated by comparing the total cleavage produced by Fe(II)·BLM A_5 in the absence of the 16-nt hairpin DNA (lane 4 in Figure 8, panels A and B) with that resulting as the concentration of the 16-nt hairpin DNA was increased (Figure 9). At a 160 μM (DNA nucleotide) concentration of the 16-nt hairpin DNA, there was no significant difference between the cleavage inhibitory effects of **1** and **2**. However, at higher concentrations of the competitor DNA, the cleavage of hairpin DNA **1** was reduced more quickly than that of **2**.

Discussion

BLM has several functional domains, including a metal binding domain, linker domain, carbohydrate moiety, and a DNA binding domain and C-terminal substituent (Figure 1). The metal binding domain is responsible for DNA binding and metal complexation as well as oxygen binding and activation.[9,16] The linker domain, bithiazole moiety, and C-terminal substituent contribute to DNA binding.[3,17] BLM binding to DNA has been suggested to occur by intercalation or through interactions with the minor groove and has been studied using a number of

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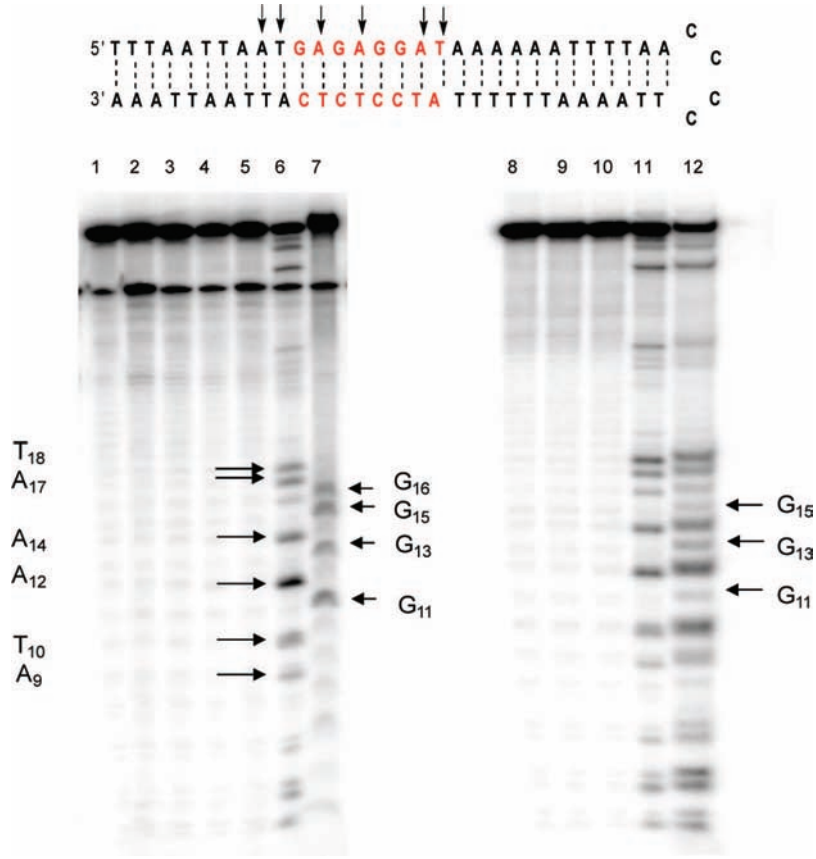

Figure 7. Sequence-selective cleavage of [5′-^{32}P]-end-labeled 64-nt hairpin DNA **4** by BLM A_5. Lane 1, radiolabeled **4** alone; lane 2, 20 μM Fe^{2+}; lane 3, 5 μM BLM A_5; lane 4, 5 μM Fe(II)·BLM A_5; lane 5, 20 μM BLM A_5; lane 6, 20 μM Fe(II)·BLM A_5; lane 7, G lane; lane 8, radiolabeled **4** alone; lane 9, 20 μM Fe^{2+}; lane 10, 20 μM BLM A_5; lane 11, 20 μM Fe(II)·BLM A_5; lane 12, 20 μM Fe(II)·BLM A_5, followed by treatment with 0.2 M *n*-butylamine.

Table 4. BLM Cleavage Sites on Hairpin DNAs **1**−**4**

	1	2	3	4
AA	+	+	+	+
AC				
AG				
AT	+++	+	+	++
CA				
CC				
CG				
CT				
GA	++	+	+	+++
GC		+		
GG				
GT		+	+	
TA			++	
TC				
TG				
TT				

different metallobleomycins, including Fe(II)·CO·BLM,[18] Zn(II)·BLM,[10b,c,19] and Co(III)·BLM.[10a,d−i,11] Further, there is accumulating evidence that there may be more than one mode of DNA interaction possible for the C-terminus of metallo-BLMs.[11,12] In contrast, all studies of DNA binding by BLMs are in agreement that the metal binding domain of metalloBLMs resides in the minor groove of DNA,[10,11] which is fully consistent with the minor groove chemistry that leads to BLM-mediated DNA degradation.

While DNA binding has been studied using spectroscopic tools, and recently by X-ray crystallography,[11] with few exceptions biochemical studies of BLM−DNA interaction have employed oxidative DNA cleavage as an end point. One biochemical study that carefully measured DNA binding by Co·BLM using DNase footprinting, as well as cleavage of both DNA strands by Fe·BLM and Co·BLM, concluded that both metalloBLMs likely bound to the same sites.[20] The finding that the cleavage of the DNA by Fe·BLM occurred only within the regions shown to involve binding via footprinting, while that of Co·BLM also occurred in A/T-rich regions, was interpreted in terms of different chemistries employed by the two metalloBLMs. In fact, Co·BLM has been reported to produce only one of the two types of chemical products formed by Fe·BLM.[21] In order to gain insight into DNA binding preferences by BLM, we recently described a SELEX-type procedure that can be used to identify hairpin DNAs that bind strongly to BLM.[13] Presently, we employ that technique to identify and characterize 10 DNAs that bind BLM strongly, and to facilitate an analysis of the structural elements in DNA required for efficient DNA binding and cleavage. Since it is known that the rate-limiting step in

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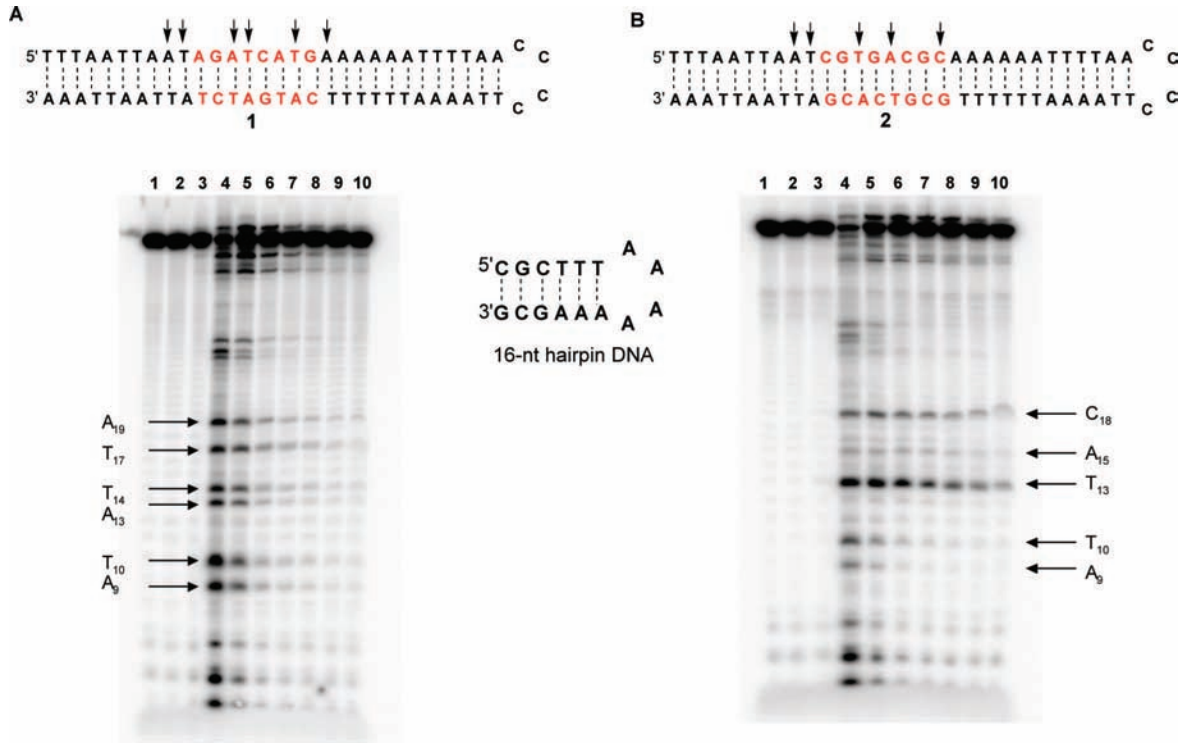

Figure 8. Competition effects of unlabeled 16-nt hairpin DNA on Fe(II)·BLM A_5-mediated cleavage of [5′-^{32}P]-end-labeled hairpin DNAs **1** and **2**. (A) Lane 1, 640 μM (nucleotide concentration) radiolabeled DNA **1** alone (64 nt); lane 2, 20 μM Fe^{2+}; lane 3, 20 μM BLM A_5; lane 4, 20 μM Fe(II)·BLM A_5; lanes 5−10, 20 μM Fe(II)·BLM A_5 + 160, 320, 640, 1280, 2560, and 5120 μM (nucleotide concentrations) unlabeled 16-nt hairpin DNA, respectively. (B) Lane 1, 640 μM (nucleotide concentration) radiolabeled DNA **2** alone (64 nt); lane 2, 20 μM Fe^{2+}; lane 3, 20 μM BLM A_5; lane 4, 20 μM Fe(II)·BLM A_5; lanes 5−10, 20 μM Fe(II)·BLM A_5 + 160, 320, 640, 1280, 2560, and 5120 μM (nucleotide concentrations) unlabeled 16-nt hairpin DNA, respectively.

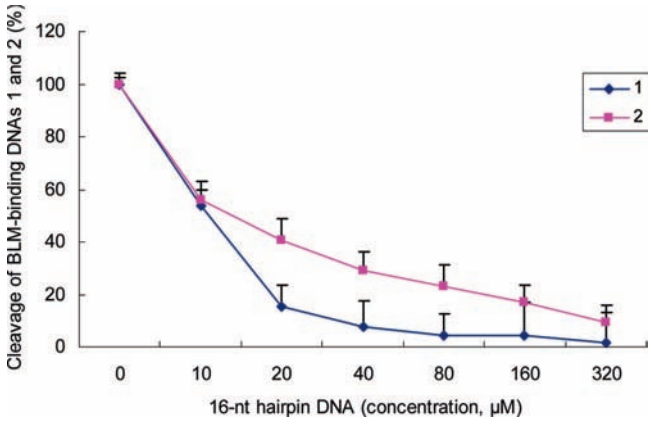

Figure 9. Comparison of the effects of unlabeled 16-nt hairpin DNA on the cleavage of [5′-^{32}P]-end-labeled hairpin DNAs **1** and **2** by Fe(II)·BLM A_5. The cleavage produced by Fe(II)·BLM A_5 on DNAs **1** and **2** in the absence of the 16-nt hairpin DNA was defined as 100%.

BLM-mediated DNA degradation is H atom abstraction[8] rather than BLM activation[7] or DNA binding per se, it seemed possible that DNAs selected for tight binding to BLM might not exhibit efficient DNA cleavage, or even cleavage at the same sites as those DNAs shown previously to be cleaved efficiently by metalloBLMs.

The selection strategy outlined in Scheme 1 relies on the ability of BLMs tethered to solid supports to interact normally with DNA substrates. In fact, this has been demonstrated using natural BLMs conjugated to activated supports[22] and also following synthesis of (deglyco) BLMs on solid supports.[12c,23] Also essential to the generation of a library containing numerous potential binding substrates for BLM was the use of hairpin DNAs. Several earlier studies have established that hairpin DNAs can be cleaved quite efficiently by metalloBLMs.[12b,13,14,24,25] The DNA library was prepared by DNA polymerase (Klenow fragment)-mediated polymerization through a single-stranded template containing an 8-nt region that had been randomized in the initial chemical synthesis of the 41-nt substrate. The completed library was thus designed to contain 4^8 (= 65 536) different sequences within the duplex region encompassing nucleotides 11−18 (and the complementary nucleotides 47−54). While not large in comparison to the number of sequences often used to initiate selections, this seemed sufficiently large to ensure a reasonable initial sampling of DNAs capable of binding strongly to BLM. In comparison with the SELEX protocol normally used,[26] it may be noted that iterative selections under increasingly stringent conditions were purposefully avoided, as the goal of the present study was the identification and characterization of a broader range of DNA sequences conducive to strong BLM binding. The procedure shown in Scheme 1 was used to identify 10 hairpin DNAs, all of which proved to bind BLM quite strongly.

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(27) In Figure 5, the polyacrylamide gel was not run as long as for the other gels. Therefore, cleavage at sites 5′-AA_9-3′ and 5′-AT_{10}-3′ was not very clear. The competition experiments carried out under the identical conditions showed very strong cleavage of hairpin DNA **2** at these two sites (Figure 8B).

Interestingly, some of the sequences contain one or more 5′-GC-3′ or 5′-GT-3′ sites, which are well known to be favorable sites for BLM-mediated DNA cleavage. However, hairpin DNAs **1** and **4** lack any 5′-GC-3′ or 5′-GT-3′ site (Table 1). An analysis of the distribution of two nucleotide sequences within the initially randomized regions of the selected hairpin DNAs is shown in Table 2. While it is not clear that BLM necessarily uses a two-nucleotide recognition element, studies of DNA cleavage by BLM are generally interpreted at the level of two adjacent nucleotides, and thus Table 2 may facilitate a comparison of factors that control both DNA binding and cleavage by BLMs. All 16 possible dinucleotides were well represented within the initially randomized region of the 10 selected hairpin DNAs. While eight of the 10 hairpin DNAs contained at least one 5′-GC-3′ or 5′-GT-3′ sequence, it is interesting that 5′-GC-3′ was present in only three of the hairpin DNAs (**2**, **7**, and **10**) and was actually the least abundant dinucleotide present, although it was present multiple times in those DNAs. The most abundant dinucleotide sequences, appearing in at least six of the hairpin DNAs, were 5′-AC-3′, 5′-AG-3′, 5′-CA-3′, 5′-CT-3′, 5′-GT-3′, 5′-TA-3′, and 5′-TG-3′, only one of which (5′-GT-3′) is associated with efficient DNA cleavage. The dinucleotide sequences that appeared the most frequently in the 10 selected DNAs were 5′-TA-3′ (14 times), 5′-CG-3′ (12 times), and 5′-GT-3′ (10 times), as well as 5′-AC-3′ and 5′-AT-3′ (10 times each). The abundance of sequences containing A and T is quite striking, as AT-rich regions of DNA are cleaved poorly by Fe(II)·BLM. It is interesting, however, that such sequences were reported to be among those cleaved by light-activated Co·BLM.[20]

Previously, a 16-nt hairpin DNA (5′-CGCT$_3$A$_7$GCG-3′), a highly efficient substrate for Fe(II)·BLM having a fluorescence-quencher pair at the 5′- and 3′-ends, was employed successfully to study the kinetics[25] and enable the visualization[23,25] of BLM-mediated DNA cleavage. In the present study, a 16-nt DNA hairpin containing 4-aminobenzo[*g*]quinazolin-2-one 2′-deoxyribose at position C$_{15}$ (hairpin DNA-C$_{f15}$, Figure 2)[14] was used to evaluate the BLM binding characteristics of the identified high-affinity DNA. Since DNA cleavage events mediated by BLM are mediated from the minor groove of DNA,[3] the presence of the benzo[*g*]quinazoline heterocycles in the major groove of the DNA duplex did not affect DNA binding and cleavage by Fe(II)·BLM.[14] The hairpin DNA-C$_{f15}$ itself exhibited efficient quenching of benzo[*g*]quinazoline fluorescence due to formation of the base-paired duplex. Likewise, a solution containing BLM A$_5$ (0.72 μM) as a control did not enhance fluorescent emission, indicating a lack of cleavage in the absence of a metal ion (black curve, Figure 3). In comparison, strong fluorescence emission could be detected following treatment of the hairpin DNA-C$_{f15}$ with equimolar Fe(II)·BLM A$_5$ (0.72 μM) (red curve, Figure 3), indicating that activated Fe(II)·BLM mediated efficient hairpin DNA-C$_{f15}$ cleavage and release of the fluorescent nucleobase. Figure 3 shows the fluorescence enhancement of hairpin DNA-C$_{f15}$ mediated by BLM A$_5$ in the presence and absence of 64-nt hairpin DNA **7**. When fluorescent hairpin DNA-C$_{f15}$ was treated with a solution containing Fe(II)·BLM A$_5$ (0.72 μM) and equimolar 64-nt hairpin DNA **7**, cleavage of the fluorescent hairpin DNA-C$_{f15}$ was suppressed, resulting in much less fluorescence enhancement (blue curve, Figure 3). The data indicate that Fe(II)·BLM A$_5$ was bound tightly to 64-nt hairpin DNA **7**, precluding degradation of the profluorescent hairpin DNA. As expected, the initial random pool of hairpin DNAs had only a limited effect in inhibiting Fe·BLM-mediated fluorescence enhancement (green curve, Figure 3). In comparison, fluorescence enhancement upon treatment with Fe(II)·BLM was significantly inhibited by all the selected hairpin DNAs (Figure 3 and Table 3).

Quantification of the inhibition of fluorescence afforded the binding specificity[13] of these 10 selected hairpin DNAs (Table 3). Hairpin DNAs **2** and **7** exhibited about 97% binding. Interestingly, hairpin DNAs **2** and **7** contain the 5′-GC-3′ and 5′-GT-3′ sequences that are the most typical strong cleavage sites for Fe(II)·BLM. The apparent equilibrium binding constants (*K*) of BLM A$_5$ determined for the selected hairpin DNAs, using the 16-nt hairpin DNA-C$_{f15}$,[13,14] agreed well with the fluorescence inhibition assay,[13] arguing that hairpin DNAs **2** and **7** actually do bind BLM the most strongly. Interestingly, the lack of 5′-GC-3′ or 5′-GT-3′ sequences in hairpin DNAs **1** and **4** did not result in the lowest binding specificity. Instead DNA **3**, having one 5′-GT-3′ sequence, exhibited the lowest binding specificity (76%, Table 3).

To permit a comparison of DNA binding and cleavage preferences for the selected hairpin DNAs, four of the DNAs (**1**−**4**) were 5′-^{32}P end-labeled and subjected to treatment with Fe(II)·BLM A$_5$ (Figures 4−7). The analysis of cleavage specificity was limited to the 5′-ends of the hairpin DNAs in the present study.

Unsurprisingly, strong cleavage bands were found for substrate **2** at 5′-GC-3′ and 5′-GT-3′ sites, which showed the strongest binding specificity (97%). These bands were readily apparent at both 5 and 20 μM Fe(II)·BLM A$_5$ (Figure 5). A 5′-GA-3′ site was also cleaved, albeit inefficiently. For hairpin DNA **3**, having one 5′-GT-3′ site and a binding specificity of only 76% (Tables 1 and 2), six strong cleavage bands were apparent, including the 5′-GT$_{15}$-3′ site (Figure 6). Hairpin DNA **1**, containing one 5′-GA-3′ site, and hairpin DNA **4**, containing two 5′-GA-3′ sites, lack 5′-GC-3′ or 5′-GT-3′ sites but were good substrates for cleavage by BLM A$_5$ (Figures 4 and 7). All of the 5′-GA-3′ sites in **1** and **4** underwent efficient cleavage. The pattern and intensities of cleavage at these sites were not consistent with the usual preference for 5′-GC-3′ and 5′-GT-3′ cleavage observed in randomly chosen DNA sequences. In addition, the extent of cleavage is not parallel to the binding specificity for the sequences. Surprisingly, 5′-AA$_9$-3′ and 5′-AT$_{10}$-3′ were also cleaved in all selected hairpin DNAs, as was 5′-GA-3′ (Figures 4−7, Table 4).[27]

As noted above, the selected hairpin DNAs were surprisingly AT-rich; the finding that 5′-AT-3′ was also cleaved in hairpin DNAs **1**−**4** suggests that AT-rich regions may be important recognition elements for Fe·BLM, albeit not efficient cleavage sites in comparison with 5′-GT-3′ and 5′-GC-3′ when the latter two are present. This is fully consistent with the earlier findings that bithiazoles[12a,28] and metalloBLMs[12a,29] can bind effectively to AT-rich DNA regions, and that light-activated Co·BLM effected DNA cleavage in A-rich regions,[20] possibly due to an altered chemistry of DNA cleavage by this metalloBLM. It is also consistent with the present finding that hairpin DNA **2**, containing four 5′-GT-3′ and 5′-GC-3′ sequences, was the only substrate in the present study cleaved to a significant extent at 5 μM Fe(II)·BLM A$_5$ concentration. It may be noted that AT-rich regions contain narrow minor grooves and are generally

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associated with DNA groove binders rather than intercalators.[30] It has been shown previously that the efficiency of DNA cleavage by Fe·BLM is very sensitive to the dimensions of the minor groove.[31] In the aggregate, the results suggest that there may well be regions of DNA bound well by Fe·BLM but not cleaved as efficiently as the well-characterized lesions involving 5′-GC-3′ and 5′-GT-3′ sequences.

In order to determine whether binding specificity (Table 2) would correlate with efficiency of cleavage if an excess of other substrate DNAs was present, the cleavage of hairpin DNAs **1** and **2** was carried out in the presence of increasing concentrations of the unlabeled hairpin DNA shown in Figure 8, the latter of which is known to be an efficient substrate for Fe·BLM.[14,25] As shown in Figures 8 and 9, the cleavage of **1** and **2** was reduced significantly in the presence of unlabeled 16-nt hairpin DNA in a concentration-dependent manner (Figure 9). Comparison of inhibitory effects on the cleavage of **1** to **2** indicated that hairpin DNA **2** (binding specificity 97%) was cleaved more efficiently than **1** (binding specificity 82%) by Fe(II)·BLM A_5 as the concentration of the competitor DNA was increased.

Although quite preliminary, one of the more intriguing observations made in the present study concerns the chemistry of DNA degradation. It has been well documented that Fe·BLM-mediated DNA degradation occurs via two pathways, the ratio of which can be affected by O_2 tension.[3] Under ambient conditions, frank strand scission is the predominant pathway, lending to the concomitant release of base propenals. The alternative pathway, which generally becomes more pronounced at lower oxygen tension, involves the formation of an alkali-labile lesion with the release of free nucleobases. The alkali-labile lesions, as the name implies, can be cleaved with alkali,[15] and also with reagents such as hydrazine and *n*-butylamine.[15b,32]

In an effort to identify the chemistry of the hairpin DNA cleavage products by Fe(II)·BLM A_5, 5′-^{32}P end-labeled hairpin DNA **4** was treated with 20 μM Fe(II)·BLM A_5 and then 0.2 M *n*-butylamine. It was found that hairpin DNA **4** underwent cleavage through both pathways. As shown in lane 12 of Figure 7, upon treatment with *n*-butylamine, three new cleavage products appeared at G_{11}, $G_{13,}$ and G_{15}. The fact that bands for oligonucleotides terminating in 3′-phosphoroglycolates were not apparent at these positions prior to *n*-butylamine treatment argues that the alkali-labile lesion must have been the predominant lesion at these three positions. This appears to be true at a number of the other sites cleaved by Fe·BLM in hairpin DNA **4** as well, as may be appreciated by a comparison of bands in lanes 11 and 12 of Figure 7. The observation of enhanced formation of alkali-labile lesions under ambient O_2 conditions has been reported in a few cases previously, notably where the DNA substrate has been altered by methylation[33] or platination.[34] Hairpin DNA **4** lacks any 5′-GC-3′ or 5′-GT-3′ sequence but is nonetheless cleaved at six sites in the presence of 20 μM Fe(II)·BLM. In this context, it is worthy of note that analyses of the chemistry of DNA degradation by Fe·BLM have generally been carried out using DNAs that are initially identified by virtue of being substrates for cleavage, and which have a number of strong cleavage sites.[3,15,35] It would be interesting to know whether such sites have been the focus of DNA degradation studies by Fe·BLM precisely because frank strand scission (as compared with alkali-labile lesion formation) is a characteristic of cleavage at such sites. If this proved to be true, then the extent and pattern of DNA degradation by Fe·BLM may not be properly represented at the present time.

Experimental Procedures

Materials. Adenosine 5′-[γ-^{32}P]triphosphate (250 μCi) was purchased from PerkinElmer Life and Analytical Sciences. DNA polymerase (Klenow fragment) and restriction endonucleases *Nde*I, *Ase*I, *Eco*RI, and *Apo*I, as well as alkaline phosphatase, calf intestinal phosphatase (CIP), and T4 polynucleotide kinase, were purchased from New England Biolabs. The vector pT7 Blue was obtained from Novagen. Competent cells DH5α were purchased from Invitrogen. $Fe(SO_4)_2(NH_4)_2 \cdot 6H_2O$ was purchased from Sigma-Aldrich and was used to prepare fresh aqueous solutions for admixture to BLM A_5 immediately prior to use. Oligonucleotides including a 41-nt template containing 8 nucleotides (X) as random sequences, 5′-TTTAATTAATXXXXXXXXAAAAAATTTTAACCCCTTAAAAT-3′, and all other 16-nt and 64-nt DNAs were purchased from Integrated DNA Technology, Inc. Plasmid Midi kits were purchased from Qiagen.

Methods. The 8-nt random sequence in the 41-nt template was prepared by addition of all four nucleoside phosphoramidites to the column for one coupling reaction; this was done for the eight relevant cycles. The concentration and purity of the DNAs were determined by measuring UV absorbance at 260 nm and UV absorbance ratio at 260/280 nm using a UV/vis spectrophotometer (Perkin-Elmer Lambda Array 3840), respectively. The extinction coefficients were estimated by the nearest-neighbor approximation.[36] The concentration of BLM A_5 was calculated by using the molar absorptivity of 14 500 $M^{-1} \cdot cm^{-1}$ at 292 nm in H_2O.[12b]

The hairpin structures of the 16-nt substrates and the 64-nt hairpin DNAs were formed in an appropriate buffer solution by heating at 80 °C for 1 min and cooling the solution slowly to room temperature. Stock solutions of hairpin DNA-C_{f15} and 64-nt hairpin DNA in 10 mM sodium cacodylate buffer, pH 7.0, containing 100 mM NaCl were diluted to 30 μM (i.e., <100 μM[37]), promoting the formation of unimolecular hairpin structures.

Fluorescence experiments were performed in a microfluorescence cell (400 μL) having a path length of 1.0 cm using a Varian fluorescence spectrophotometer (Cary Eclipse) with a PCB-150 water circulator (25 °C). The data were collected using the software supplied with the instrument. The aqueous solutions were prepared using deionized and sterilized distilled water.

Synthesis of DNA Hairpin Library by Klenow Fragment. The 41-nt template containing 8-nt random sequences (10 μL, 1 μg/μL) was self-annealed at 75 °C for 15 min, and then treated with 2 μL of DNA polymerase Klenow fragment (40 U) and 10 μL of 5 mM dNTPs in 10 mM Tris-HCl, pH 7.9, containing 50 mM NaCl, 10 mM $MgCl_2$, and 1 mM DTT. The reaction mixture was incubated at 37 °C for 30 min and then heated at 75 °C for 20 min.

Binding to 64-nt Hairpin DNA by Resin-Bound BLM A_5. The resin-bound BLM A_5[12c] (0.18 μmol, 30 μmol/g) was incubated with

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0.2 nmol of 64-nt hairpin DNA in 20 μL (total volume) of 20 mM Tris-HCl buffer, pH 7.4 at room temperature for 20 min. The mixture was washed twice with 20 μL of 20 mM Tris-HCl buffer. Then the hairpin DNA still bound to BLM A_5 was isolated from the solid support by washing with 1 M NaCl and desalted by Microcon YM 10 filtration (Millipore).

Sequencing of the Selected Hairpin DNA by Subcloning Techniques. Digestion of the Selected DNAs with Restriction Enzymes *Ase*I and *Apo*I. The hairpin DNAs isolated from resin-bound BLM A_5 were digested with 30 U of restriction endonuclease *Ase*I in 50 μL of 50 mM Tris-HCl, pH 7.9, containing 100 mM NaCl, 10 mM $MgCl_2$, and 1 mM DTT. The reaction mixture was incubated at 37 °C for 1 h and then incubated with 30 U of *Apo*I at 50 °C for 1 h. The enzymes were inactivated at 80 °C for 20 min.

Digestion of pT7 Blue with *Nde*I and *Eco*RI. The plasmid vector pT7 Blue (30 μg) was digested with 20 U of *Nde*I at 37 °C for 1 h and then incubated with 20 U of *Eco*RI at 37 °C for 1 h in 50 μL of 20 mM Tris-acetate, pH 7.9, containing 50 mM potassium acetate, 10 mM magnesium acetate, and 1 mM DTT. The incubation mixture was heated at 65 °C for 15 min to inactivate the enzymes.

Dephosphorylation of the Digested Plasmid To Prevent Self-Ligation. The digested plasmid vector pT7 Blue was treated with 2 U of calf intestinal phosphatase in 50 mM Tris-HCl, pH 7.9 (100 μL total volume), containing 100 mM NaCl, 10 mM $MgCl_2$, and 1 mM DTT at 37 °C for 1 h and then worked up by phenol:chloroform extraction.

Ligation of the Oligomer to the Digested Plasmid. The selected DNA was inserted into the digested plasmid pT7 Blue via the agency of T4 DNA ligase in 50 mM Tris-HCl, pH 7.5, containing 10 mM $MgCl_2$, 10 mM DTT, and 1 mM ATP. The reaction mixture was incubated at 16 °C for 3 h.

Bacterial Transformation and Growth. To 50 μL of competent cells DH5α preparation was added 10 μL (0.05 μg) of recombinant DNA. The incubation mixture was stirred gently and maintained on ice for 30 min. The incubation mixture was then heated at 42 °C for 30 s and placed on ice for 10 min. The mixture was diluted with 1 mL of LB (10 mg/mL bacto trypton, 5 mg/mL bacto yeast extract, 10 mg/mL NaCl, pH 7.4) and incubated at 37 °C for 2 h with shaking at 150 rpm. The suspension was cultured on LB agar plates including 100 μg/mL ampicillin, 30 μg/mL X-gal, and 1 mM IPTG at 37 °C overnight. The white colonies were transferred to LB broth including ampicillin (100 μg/mL) and incubated at 37 °C overnight.

Isolation and Sequencing of Selected DNA. The isolation and purification of plasmid DNA was carried out using Qiagen plasmid Midi kits. DNA sequencing was carried out at the Biomolecular Research Facility of the University of Virginia.

5′-^{32}P Radiolabeling of Hairpin DNA and Cleavage of the End-Labeled Hairpin DNA by Fe(II)·BLM A_5. Briefly, the 64-nt hairpin DNA was [5′-^{32}P]-end-labeled with 5′-[γ-^{32}P]ATP and T4 polynucleotide kinase and purified by 20% polyacrylamide gel electrophoresis. The hairpin DNA cleavage reaction containing the 5′-^{32}P-labeled hairpin DNA (2×10^5 cpm, 0.5−5 μM) was carried out in 5 μL (total volume) of 10 mM sodium phosphate, pH 8.0, containing different concentrations of Fe(II)·BLM A_5. Each reaction was initiated by the simultaneous addition of BLM and $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ (freshly prepared solution) to the buffered solution containing the hairpin DNA. Each reaction mixture was incubated at room temperature for 30 min, and then the reaction mixture was dissolved in a gel loading buffer (98% formamide, 2 mM EDTA, 0.025% xylene FF, and 0.025% bromophenol blue). The resulting solution was heated at 70 °C for 5 min and then analyzed by 20% polyacrylamide gel (31 cm × 38.5 cm × 0.4 cm) electrophoresis at 50 W for 1.5 h. The gel was analyzed using a phosphorimager.

Fluorescence Inhibition Assay of 64-nt Hairpin DNA. A solution formed by admixture of 12 μL of 30 μM 64-nt hairpin DNA and 2.5 μL of 144 μM BLM A_5 was pre-incubated for 20 min. The solution containing the 64-nt hairpin DNA and BLM A_5 was added to a hairpin DNA-C_{f15} solution prepared by addition of 12 μL of 30 μM hairpin DNA-C_{f15} to 469 μL of 10 mM sodium cacodylate buffer, pH 7.0, containing 100 mM NaCl. The reaction mixture was maintained at room temperature for 1 min. To this solution was added 5 μL of 72 μM freshly prepared aqueous $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$. The same volume of buffer solution was added to the control sample without Fe^{2+} and 64-nucleotide hairpin DNA. The final concentrations of hairpin DNA-C_{f15}, 64-nt hairpin DNA, and Fe(II)·BLM A_5 were all 0.72 μM (total volume 500 μL). The reaction mixture was incubated at room temperature for 30 min. The fluorescence emission was measured at 25 °C. The samples were excited at 310 nm, and the emission signal was measured from 400 to 550 nm using an excitation slit width of 10 nm and an emission slit width of 10 nm.

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