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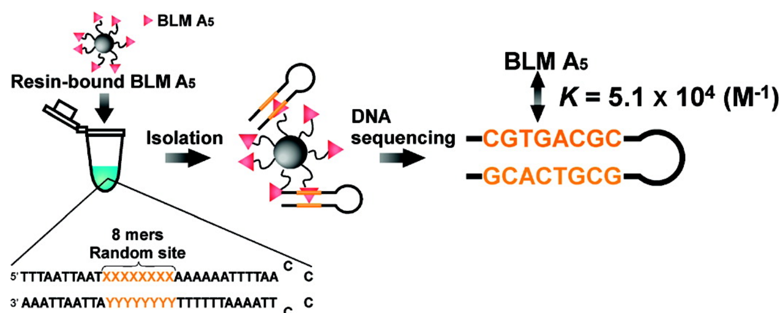

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Identification of Strong DNA Binding Motifs for Bleomycin

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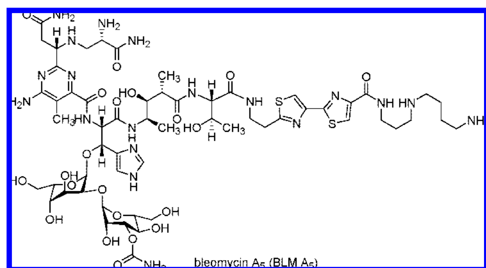
The bleomycins (BLMs) are a family of clinically used antitumor agents exemplified by BLM A_5.[1] Their mechanism of action is believed to involve oxidative cleavage of DNA and possibly also RNA. DNA degradation has been studied extensively and shown to involve binding of an activated metallobleomycin to DNA,[2] followed by abstraction of C4′-H from deoxyribose in the rate-limiting step[3] for DNA degradation.

While DNA and RNA degradation by activated Fe•BLM has been well studied,[4,5] less is known about the actual binding selectivity of BLM, that is, the obligatory step that precedes cleavage. It is unclear whether cleavage specificity is defined by the binding event or occurs at a subset of preferred binding sites. With only a few exceptions,[6] NMR binding studies have employed metalloBLMs such as Co•BLM[7] and Zn•BLM[8] whose therapeutic relevance is uncertain. A single biochemical study that compared DNA binding and cleavage directly also employed Co•BLM.[9] Herein, we describe the development and implementation of a novel strategy to identify DNA motifs that bind BLM strongly.



The strategy used to identify BLM binding motifs is shown in Scheme 1. A 41-nucleotide (nt) DNA substrate containing an 8-nt randomized sequence in positions 11−18 was converted to a 64-nt hairpin DNA containing 8 contiguous randomized base pairs via the action of DNA polymerase (Klenow fragment[10]). The mixture of hairpin DNAs was incubated with resin-bound BLM A_5[11,12] in 20 mM Tris-HCl buffer, pH 7.4, for 20 min; after washing with buffer, the bound DNA was recovered by washing the resin with 1 M NaCl and then desalted. The mixture of isolated hairpin DNAs was digested with restriction endonucleases *Ase*I and *Apo*I, then ligated into the corresponding site of predigested plasmid pT7 Blue. Following plasmid transformation into *Escherichia coli* DH5α, several colonies containing 64-nt DNA inserts were used for recovery of the amplified 64-nt hairpin DNAs. These were studied for their DNA binding characteristics (vide infra), and the hairpins of interest were sequenced (Table 1).

To evaluate the BLM binding characteristics of these DNAs, we initially employed an assay based on inhibition of BLM A_5-mediated cleavage of a 16-nt hairpin DNA having a fluorescent nucleoside at the site of cleavage. This fluorescent hairpin DNA was designed based on the sequence of a highly efficient DNA substrate for BLM[2,13] and has been shown to be degraded efficiently by BLM A_5.[14] Degradation resulted in a strong enhancement of fluorescence emission (Figure 1).[14,15]

Scheme 1. Strategy for Isolation of DNA Hairpin Motifs That Bind BLM Strongly

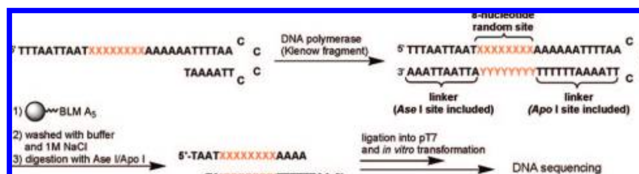


Table 1. DNA Motifs That Bound Strongly to Resin-Linked BLM A_5

5′ TTTAATTAATXXXXXXXXAAAAAATTTTAA (C C C C loop)
3′ AAATTAATTAYYYYYYYYTTTTTTAAAATT

1	5′ AGATCATG 3′ TCTAGTAC	3	5′ TAAGTGGG 3′ ATTCACCC
2	5′ CGTGACGC 3′ GCACTGCG	4	5′ GAGAGGAT 3′ CTCTCCTA

A solution containing BLM A_5 (0.72 μM) and equimolar 64-nt hairpin DNA was maintained at 25 °C for 20 min. This mixture was added to a buffered solution containing fluorescent hairpin DNA-C_{f15} and maintained at 25 °C for 1 min, followed by the addition of Fe^{2+} to initiate DNA cleavage. When Fe(II)•BLM A_5 was bound tightly to a 64-nt hairpin DNA, the cleavage of fluorescent hairpin DNA-C_{f15} was suppressed, resulting in less fluorescence enhancement. Figure 1 shows the fluorescence enhancement of hairpin DNA-C_{f15} mediated by BLM A_5 in the presence and absence of 64-nt hairpin DNAs. Treatment of hairpin DNA-C_{f15} with equimolar Fe(II)•BLM A_5 in the absence of 64-nt hairpin DNA gave strong fluorescence enhancement following excitation at 310 nm; the reaction mixture without Fe^{2+} showed almost none, indicating that activated Fe•BLM mediated hairpin DNA-C_{f15} cleavage and release of the fluorescent nucleobase. In the presence of selected hairpin DNAs **1**, **2**, **3**, or **4**, the fluorescence enhancement was significantly inhibited while the initial random mixture of hairpin DNAs had only a limited effect (Figure 1).

Analysis of binding specificity[16] indicated that DNA **2** exhibited 97% binding, essentially reducing fluorescence enhancement to the level produced in the absence of Fe^{2+}. The remaining three hairpin DNAs (**1**, **3**, and **4**) had binding specificities <75%, in comparison with the initial randomized pool of 64-nt hairpin DNAs (specificity ∼25%) (Figure S1, Supporting Information).[17] Interestingly, **1** and **4** lack 5′-GC-3′ or 5′-GT-3′ sequences that are the most typical cleavage sites for Fe(II)•BLM.

To assess the dynamics of BLM binding by the selected hairpin DNAs, a different experimental protocol was employed. A solution of 0.72 μM BLM A_5 and equimolar fluorescent hairpin DNA-C_{f15} was preincubated at 25 °C for 20 min. This solution was then added

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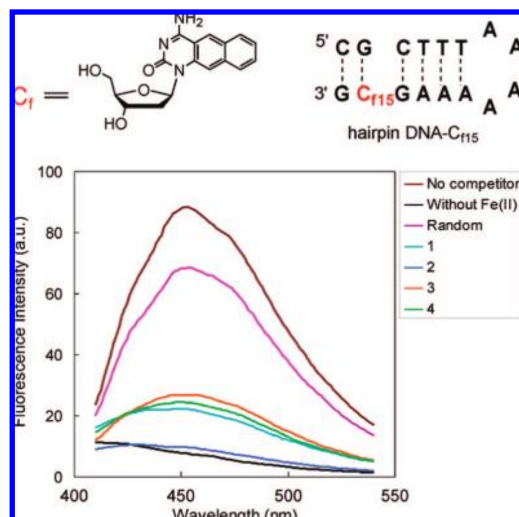

Figure 1. Emission spectra of hairpin DNA-C_{f15} (0.72 μM, having the 2′-deoxyriboside of 4-aminobenzo[*g*]quinazoline-2-one (C_f) at position 15) treated with 0.72 μM Fe(II)•BLM A_5 (brown) and 0.72 μM BLM A_5 (black) after 30 min incubation at 25 °C in 10 mM sodium cacodylate buffer, pH 7.0, containing 100 mM NaCl (λ_{ex} 310 nm). The emission spectra of hairpin DNA-C_{f15} treated with Fe(II)•BLM A_5 in the presence of equimolar **1** (light blue), **2** (blue), **3** (orange), and **4** (green) under the same conditions are also shown to compare with 64-nt hairpin DNA containing a random sequence of 8 base pairs (magenta) as a control.

Table 2. Apparent Equilibrium Constants of BLM A_5 to Hairpin DNA[a]

hairpin DNA	apparent equilibrium constrants ($K \times 10^4$) (M^{-1})
16-mer DNA[b]	1.7 ±0.16
2	5.1 ±0.56
random[c]	N.D.

[a] With 10 mM Tris-HCl buffer solution, pH 8.4. [b] With 16-nt hairpin DNA, 5′-CGCTTTAAAAAAAGCG-3′. [c] Not determined.

to a solution containing the same quantity of 64-nt hairpin DNA **2** in buffer. The combined solution was maintained at 25 °C for 1 min, followed by the addition of Fe^{2+} (Figure S2, Supporting Information). Fluorescence enhancement was completely suppressed, indicating that the BLM A_5 prebound to the fluorescent hairpin DNA became rebound to hairpin DNA **2** within 1 min. These results strongly support the conclusion that the isolated hairpin DNAs bind Fe•BLM A_5 much more strongly than fluorescent hairpin DNA-C_{f15}, which has been shown to be a very effective substrate for Fe•BLM.[14]

To determine apparent equilibrium binding constants (*K*) of BLM A_5 for the selected hairpin DNAs, fluorescence quenching based on DNA binding of BLM was carried out.[18] The *K* values of BLM A_5 for the 16-nt hairpin DNA and **2** in 10 mM Tris-HCl buffer, pH 8.4, were 1.7×10^4 and 5.1×10^4 M^{-1}, respectively (Table 2). In contrast, the affinity of the 64-nt hairpin DNA containing a random sequence of 8 base pairs could not be determined due to extremely limited quenching of bithiazole fluorescence. This was in good qualitative agreement with the fluorescence inhibition assay (cf Figure 1), but the strong inhibition of BLM A_5-mediated degradation of the fluorescent hairpin DNA suggests that the *K* value determined for **2** may significantly understate the actual BLM affinity of this DNA.[18]

The finding of strong BLM binding by a hairpin DNA (**2**) containing 5′-GT-3′ and 5′-GC-3′ sites raises the question of whether this DNA is also cleaved at those sites by Fe•BLM A_5. In fact, the anticipated cleavage of 5′-GT_{13}-3′ and 5′-GC_{18}-3′ was observed by polyacrylamide gel analysis using 5′-^{32}P end labeled **2** as a substrate (Figure S3, Supporting Information).

These experiments demonstrate the feasibility of identifying DNAs containing strong BLM binding sites from a hairpin DNA library. While iterative selections under increasingly stringent conditions[21] were purposefully not used, each of several isolated sequences indicated enhanced BLM binding. Straightforward extension of this strategy can be envisioned to enable measurement of the effects on DNA binding resulting from defined changes in BLM structure.

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Supporting Information Available: Experimental procedures for isolation of 64-nt hairpin DNAs, fluorescent inhibition assay, and determination of *K* values as well as details of the DNA binding characteristics of BLM and BLM-mediated DNA cleavage. This material is available free of charge via the Internet at http://pubs.acs.org.

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(15) The extent of DNA cleavage by different BLMs (PAGE assay) versus intensity of fluorescence enhancement obtained with the fluorescent hairpin DNA indicated very similar cleavage efficiencies in both assay systems.
(16) 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%) (Figure 1).
(17) While the initial experiments were carried out using hairpin DNAs isolated as outlined in Scheme 1, detailed characterization employed chemically synthesized DNAs prepared to verify the initial observations.
(18) Fluorescence emission of the BLM bithiazole moiety is quenched by association with B-form DNA, providing a quantitative method to determine the *K* value.[19,20] This method requires the assumption that bithiazole fluorescence quenching bears the same proportionality to DNA binding for all DNAs. The rather different efficiencies of quenching of bithiazole fluorescence by DNAs shown functionally to bind BLM with comparable affinities (Figure 1) suggests that this assumption is suspect.
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