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DNA Methylation Reduces Binding and Cleavage by Bleomycin

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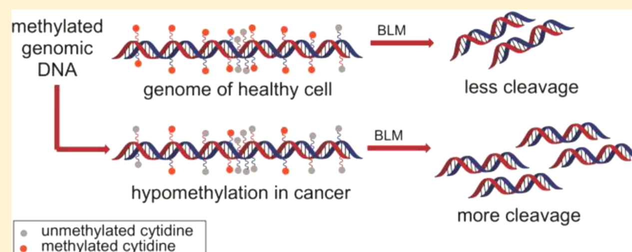
Supporting Information

ABSTRACT: In a recent study, we described the enhanced double-strand cleavage of hairpin DNAs by Fe·bleomycin (Fe·BLM) that accompanies increasingly strong binding of this antitumor agent and suggested that this effect may be relevant to the mechanism by which BLM mediates its antitumor effects. Because the DNA in tumor cells is known to be hypomethylated on cytidine relative to that in normal cells, it seemed of interest to study the possible effects of methylation status on BLM-induced double-strand DNA cleavage. Three hairpin DNAs found to bind strongly to bleomycin, and their methylated counterparts, were used to study the effect of methylation on bleomycin-induced DNA degradation. Under conditions of limited DNA cleavage, there was a significant overall decrease in the cleavage of methylated hairpin DNAs. Cytidine methylation was found to result in decreased BLM-induced cleavage at the site of methylation and to result in enhanced cleavage at adjacent nonmethylated sites. For two of the three hairpin DNAs studied, methylation was accompanied by a dramatic decrease in the binding affinity for Fe·BLM, suggesting the likelihood of diminished double-strand cleavage. The source of the persistent binding of BLM by the third hairpin DNA was identified. Also identified was the probable molecular mechanism for diminished binding and cleavage of the methylated DNAs by BLM. The possible implications of these findings for the antitumor selectivity of bleomycin are discussed.



The bleomycins [BLMs (Figure 1)] make up a family of glycopeptide-derived antitumor agents employed clinically for the treatment of several types of cancer.[1−3] Their antitumor activity has been attributed to their well-characterized sequence selective cleavage of DNA.[4−7] Although they mediate efficient single-strand DNA cleavage, their antitumor activity has been thought to be due to their ability to mediate specific double-strand DNA cleavage,[8,9] and a recent study has suggested that the nature of double-strand cleavage may be a strong function of the affinity of BLM for specific DNAs.[10]

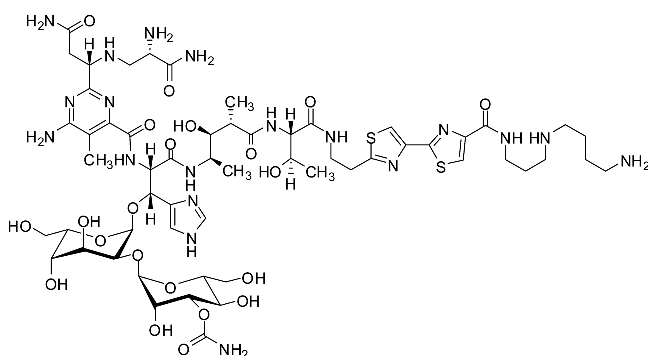


Figure 1. Structure of bleomycin A_5.

Cytidine methylation is a key factor in epigenetic gene regulation as well as carcinogenesis. Characterized by its dynamic nature,[11] the DNA methylation pattern is altered in cancer cells and marked by overall hypomethylation,[12−16] although local CpG-cytidine hypermethylation has been documented in a number of cancers,[14,16] especially with regard to CpG islands in tumor suppressor regions.[17−19] Given the observed effect of DNA structure on double-strand cleavage by BLM,[10] and earlier reports suggesting an effect of methylation on DNA cleavage by BLM,[20,21] it seemed of interest to determine whether DNA methylation might also affect double-strand cleavage and potentially provide an additional mechanism for selective cleavage of DNA in tumor cells.

In recent studies from our laboratory, the use of hairpin DNAs that bound strongly to BLM A_5 revealed enhanced double-strand cleavage,[10,22] which occurred both by the previously reported coupled double-strand cleavage mechanism[9,23] and by a novel mechanism involving two closely spaced independent single-strand breaks.[10] In this study, we employ three strongly bound hairpin DNAs (Figure 2) to study the effects of DNA methylation on their interaction with Fe(II)·BLM A_5. We also demonstrate the probable molecular basis for diminished binding and cleavage of methylated DNAs by Fe(II)·BLM.

MATERIALS AND METHODS

T4 polynucleotide kinase was purchased from New England Biolabs. Recombinant terminal deoxynucleotidyltransferase was

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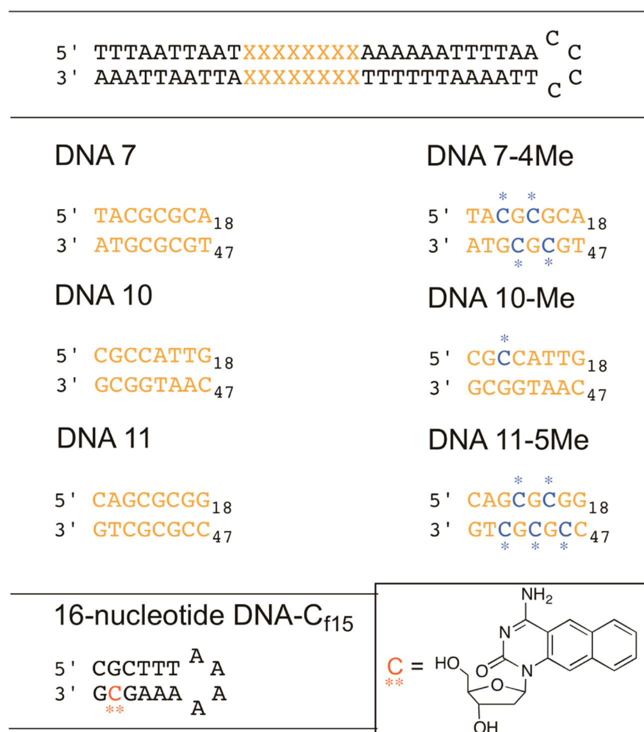

Figure 2. Three 64-nucleotide hairpin DNAs, their methylated counterparts, and a 16-nucleotide profluorescent hairpin DNA[46] employed in a competition assay with the 64-nucleotide hairpin DNAs. The blue base is 5-methylcytidine.

obtained from Roche Applied Science. Radiolabeled nucleotides, [γ-^{32}P]ATP and [α-^{32}P]cordycepin, were purchased from PerkinElmer Life Sciences. $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ and Chelex 100 were obtained from Sigma-Aldrich. Bleomycin A_5 was obtained as an outdated clinical sample. All synthetic oligonucleotides, including the hairpin DNAs, were purchased from Integrated DNA Technologies, Inc.

5′- and 3′-^{32}P End Labeling and Purification of Hairpin DNAs.[10] The hairpin DNAs were ^{32}P-end-labeled using a combination of [γ-^{32}P]ATP with T4 polynucleotide kinase and [α-^{32}P]cordycepin with terminal deoxynucleotidyltransferase for labeling at the 5′- and 3′-ends, respectively. Ten picomoles of 64-nucleotide hairpin DNAs was 5′-^{32}P-end-labeled by incubation with 20 units of T4 polynucleotide kinase and 0.06 mCi of [γ-^{32}P]ATP [specific activity of 6000 Ci (222 TBq)/mmol] in 50 μL (total volume) of 70 mM Tris-HCl buffer (pH 7.6) containing 10 mM $MgCl_2$ and 5 mM DTT. The reaction mixture was incubated at 37 °C for 1 h followed by purification of the labeled DNAs by 16% polyacrylamide gel electrophoresis at 1800 V for 2.5 h. The 3′-^{32}P end labeling was conducted by incubating 10 pmol of hairpin DNA with 20 units of terminal deoxynucleotidyltransferase and 0.06 mCi of [α-^{32}P]cordycepin [specific activity of 6000 Ci (222 TBq)/mmol] in 50 μL (total volume) of 70 mM Tris-HCl buffer (pH 7.6) containing 10 mM $MgCl_2$, 10 mM $CoCl_2$, and 5 mM DTT. The reaction mixture was incubated at 37 °C for 1 h followed by purification of DNA by 16% polyacrylamide gel electrophoresis at 1800 V for 2.5 h.

Double-Strand DNA Cleavage of 5′- and 3′-^{32}P-End-Labeled Hairpin DNAs by Bleomycin A_5.[10] Bleomycin-mediated cleavage of 5′- and 3′-^{32}P-end-labeled hairpin DNAs was performed by incubating the hairpin DNA (~30000 cpm) with 5 μM Fe^{2+} and 5 μM bleomycin A_5 at 25 °C for 30 min in 10 mM sodium cacodylate (pH 7.0, total volume of 5 μL) containing 10 μL of 2 mM $MgCl_2$. Two microliters of native gel loading buffer containing 0.25% bromophenol blue, 0.25% xylene cyanol, and 40% D-sucrose was added to the reaction mixture, which was separated on a 20% native polyacrylamide gel (200 V, 16 h, 4 °C). Double-strand cleavage sites were identified by visualizing comigrating bands using a phosphorimager.

Maxam−Gilbert Sequencing Reaction.[24] Ten microliters of 5′- and 3′-^{32}P-end-labeled DNAs (~50000 cpm) was incubated with 25 μL of formic acid at 25 °C for 5 min. The reaction mixtures were combined with 200 μL of 0.3 M sodium acetate, containing 0.1 mM EDTA, and 25 μg/mL *Escherichia coli* tRNA. The resulting solutions were immediately mixed with 750 μL of ethanol to precipitate the DNAs. The DNA pellets were washed twice with 70% ethanol and then resuspended in 75 μL of 10% piperidine. The reaction mixtures were incubated at 90 °C for 30 min and then chilled on ice. The cooled supernatants were concentrated under diminished pressure. The DNA pellets were washed with small amounts of water to remove residual piperidine and mixed with denaturing loading buffer containing 80% formamide, 2 mM EDTA, 1% bromophenol blue, and 1% xylene cyanol. The combined solutions were heated at 90 °C for 10 min and used as the sequencing lanes to compare 5′- and 3′-^{32}P-end-labeled DNAs on denaturing polyacrylamide gels.

Sequence Selective Cleavage of Radiolabeled Hairpin DNA by BLM A_5. A 5′-^{32}P-end-labeled hairpin DNA (~50000 cpm) was incubated with 5 or 10 μM Fe(II)·BLM A_5 in a 5 μL reaction mixture containing 10 mM sodium cacodylate buffer (pH 7.0) for 30 min. Five microliters of denaturing gel loading buffer containing 98% formamide, 2 mM EDTA, 0.25% (w/v) bromophenol blue, and 0.25% (w/v) xylene cyanol was added to the reaction mixture. The resulting solution was heated at 90 °C for 10 min and then chilled on ice. Five microliters of each sample was loaded onto a denaturing gel (16% polyacrylamide and 7 M urea) along with 2 μL of 5′- and 3′-^{32}P-end-labeled Maxam−Gilbert sequencing lanes to determine the sequences of the cleavage sites. The gel was run at 50 W for 2.5 h. The gels were visualized using a phosphorimager.

Fluorescence Competition Analysis of Binding of BLM to Hairpin DNAs. The 64-nucleotide hairpin DNAs (5 μM) were incubated with 5 μM bleomycin A_5 in a 10 mM sodium cacodylate buffer solution for 20 min at room temperature (25 °C). The resulting solution was mixed with 5 μM 16-nucleotide hairpin DNA having C_f in lieu of cytidine15 (hairpin DNA-C_{f15}) and incubated at room temperature for 1 min. Freshly prepared $Fe(NH_4)_2(SO_4)_2$ was added to the solution to a final concentration of 1 μM. The final solution contained 16-nucleotide hairpin DNA, 64-nucleotide hairpin DNA, bleomycin A_5, and Fe^{2+}, all at concentrations of 1 μM. The combined solution was maintained at room temperature for 30 min. The same volume of buffer solution was added to the control samples without Fe^{2+} or 64-nucleotide hairpin DNA. The fluorescence emission was measured at 25 °C. The samples were excited at 310 nm, and the emission signal was recorded from 400 to 550 nm using an excitation slit width of 10 nm and an emission slit width of 10 nm.

■ RESULTS

DNA Methylation Results in Reduced Cleavage by Fe·BLM. Comparison of the Fe(II)·BLM A_5-induced cleavage of

5′-^{32}P-radiolabeled DNAs **7** and **10** (Figure 2) with their methylated counterparts is shown in Figure 3 under conditions

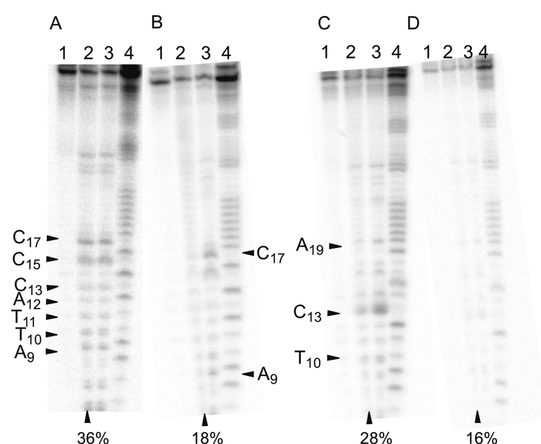

Figure 3. (A) Sequence selective cleavage of DNA **7** by BLM A_5: lane 1, DNA **7** alone; lane 2, 5 μM Fe(II)·BLM A_5; lane 3, 10 μM Fe(II)·BLM A_5; lane 4, G+A lane. (B) Sequence selective cleavage of DNA **7-4Me** by BLM A_5: lane 1, DNA **7-4Me** alone; lane 2, 5 μM Fe(II)·BLM A_5; lane 3, 10 μM Fe(II)·BLM A_5; lane 4, G+A lane. (C) Sequence selective cleavage of DNA **10**: lane 1, DNA **10** alone; lane 2, 5 μM Fe(II)·BLM A_5; lane 3, 10 μM Fe(II)·BLM A_5; lane 4, G+A lane. (D) Sequence selective cleavage of DNA **10-Me**: lane 1, DNA **10-Me** alone; lane 2, 5 μM Fe(II)·BLM A_5; lane 3, 10 μM Fe(II)·BLM A_5; lane 4, G+A lane. The numbers at the bottom of lanes 2 represent the total percent cleavages of the DNAs in those lanes.

of limited DNA cleavage, as evidenced by the persistence of uncleaved DNA in each lane. As is readily apparent, a decrease in the intensity of the cleavage bands was observed for both methylated DNAs. At 5 μM Fe(II)·BLM A_5, cytidine methylation reduced overall cleavage of DNA **7** from 36 to 18%, while for DNA **10**, methylation of a single cytidine (C_{13}) reduced the cleavage from 28 to 16%. A comparable effect was noted for hairpin DNA **11** [from 32 to 17% (Figure S1 of the Supporting Information)]. The statistical significance of the diminution in cleavage was verified by conducting the cleavage reactions in replicate experiments, and the results are summarized in Table 1, which indicates an ~2-fold reduction in cleavage for each. Interestingly, while all of the Fe·BLM cleavage bands for DNA **7** appeared to be diminished upon methylation of cytidines at four positions (cf. panels A and B of Figure 3), cleavage at sites more distant from the sites of methylation (e.g., A_9 and C_{17}) seemed to diminish less. This

Table 1. Percentage of DNA Cleavage of Hairpin DNAs[a]

hairpin DNA	percent cleavage (%)
DNA **7**	35 ± 3
DNA **7-4Me**	16 ± 2
DNA **10**	26 ± 4
DNA **10-Me**	16 ± 3
DNA **11**	33 ± 3
DNA **11-5Me**	15 ± 4
DNA **9**	20 ± 1
DNA **9-2dU**	27 ± 2

[a]The percent cleavage (%) for 5 μM Fe·bleomycin treatment was calculated as the ratio of the intensity of cleavage bands to the overall intensity of the DNA hairpin loaded in each well of a denaturing polyacrylamide gel.

effect was documented more directly by recording the intensity of cleavage at each position from A_9 through C_{17} following cleavage with 10 μM Fe(II)·BLM A_5. As shown in Figure 4A,

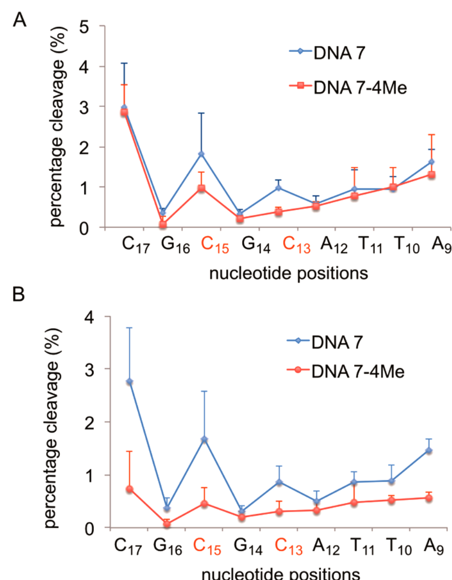

Figure 4. Percent DNA cleavage of DNA **7** and DNA **7-4Me** at nucleotide positions A_9–C_{17} following treatment with (A) 10 μM Fe(II)·BLM A_5 or (B) 5 μM Fe(II)·BLM A_5.

methylation caused a decrease in total DNA cleavage, largely as a result of diminished cleavage at the sites of the methylated cytidines. Analysis of cleavage at the same sites following treatment with 5 μM Fe(II)·BLM A_5 revealed suppressed cleavage essentially at all sites (Figure 4B).

Characterization of Double-Strand Cleavage of DNAs. As summarized in Figure 5 on the basis of results from four laboratories,[8,9,23,25−31] the double-strand cleavage of DNA involves initial abstraction of a C-4′ H atom from a DNA sugar, leading to an initially formed C-4′ deoxyribose radical.[6,7,32−34] That intermediate can partition to form either of two sets of products, one of which leads to a C-4′ hydroxyapurinic acid without immediate strand scission (Figure 5, product I),[32−42] although this species is alkali labile and can undergo subsequent strand scission.[42,43] The second set of products results in frank strand scission (Figure 5, product II).[32−39,44] For the second set of products, it has been shown that a coupled double-strand cleavage can occur at a site on the strand opposite the initial strand break. Again, two products can form: the appearance of a C-4′ hydroxyapurinic acid on the second leads to product III (Figure 5) without the direct formation of a double-strand break, while frank strand scission leads to a double-strand break (Figure 5, product IV). Because double-strand breaks of type IV have been observed under conditions of single-hit kinetics,[9] it is believed that they result from reactivation of the same DNA-bound bleomycin that induced the initial lesion on the first strand. In fact, such double-strand breaks afford well-defined double-strand cleavage patterns with regard to sequence selectivity (the first cleavage almost always occurs at a GPy sequence) and the relative position of the second cleavage (which generally affords a duplex with a blunt end or single-base 5′-extension).[9]

We have recently analyzed the nature of double-strand cleavage of 10 hairpin DNAs strongly bound by bleomycin.[10] While coupled double-strand cleavage was also noted for these

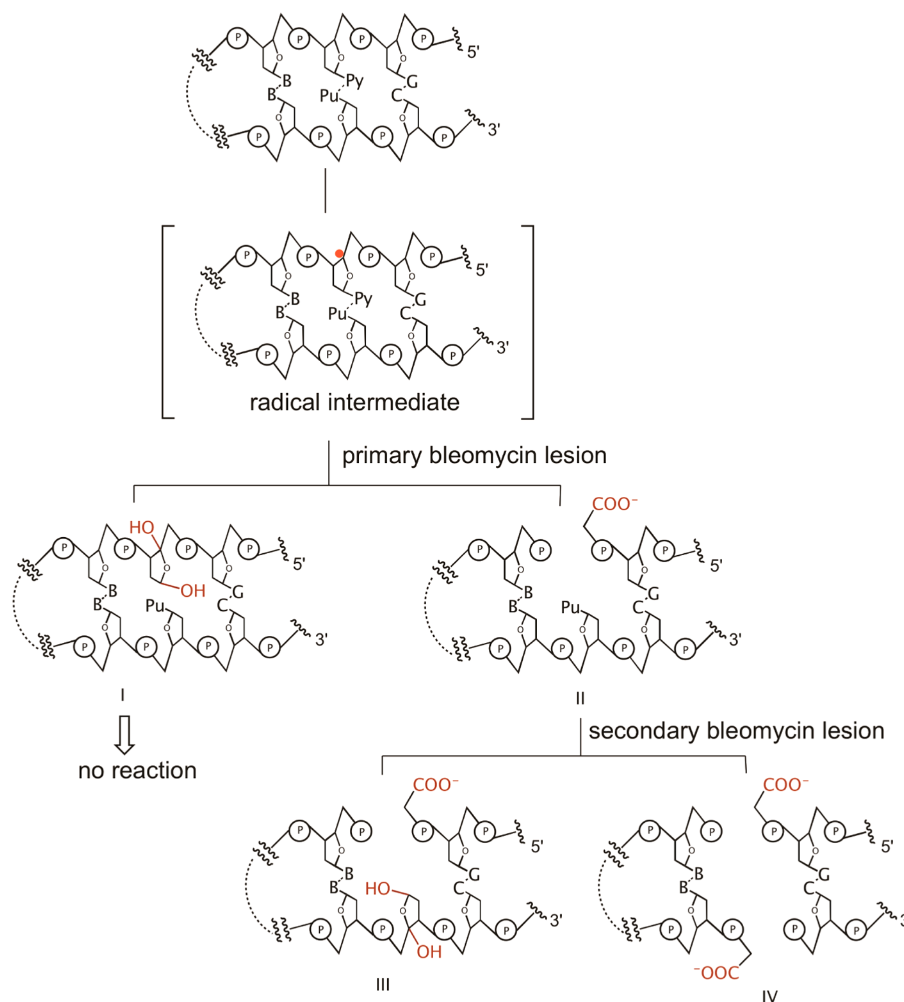

Figure 5. Mechanisms of bleomycin-induced double-strand DNA cleavage. Activated Fe·BLM abstracts a H atom from the C-4′ position of deoxyribose at the primary site (typically 5′-G-Py-B-3′ of one strand of the hairpin DNA, where B is any nucleobase) producing either an AP (apyrimidinic/apurinic) site (I) or a single-strand break terminating with a 3′-phosphoroglycolate (II) from the initially formed C-4′ deoxyribose radical intermediate shown in brackets. While the AP site does not undergo further reaction, strand break II is a potential target for a secondary bleomycin cleavage on the opposing strand of the hairpin DNA. The secondary attack of (re)activated bleomycin, by abstracting the C-4′ H atom from the secondary site (5′-B-Pu-C-3′ of the bottom strand of the hairpin DNA), affords either a strand break with a 5′-phosphate and 3′-phosphoroglycolate or an AP site. The final products are either a double-strand break with 5′-phosphate and 3′-phosphoroglycolate termini (IV) or a strand break at the primary site accompanied by an AP site at the secondary site (III), which upon treatment with mild base (e.g., *n*-butylamine) produces a double-strand cleavage product.

hairpin DNAs, the majority of the observed double-strand breaks resulted from two independent breaks; these could be distinguished from the coupled double-strand breaks by analysis of products of type III (Figure 5) and also generally exhibited sequence selectivity different from those found for coupled double-strand breaks.[10]

The double-strand cleavage of hairpin DNA **11** was conducted as described previously[10] by treating the alternatively 5′- and 3′-^{32}P-end-labeled hairpin DNA with Fe·BLM A_5 and then analyzing the products on a nondenaturing polyacrylamide gel (Figure 6A). The comigrating bands in lanes 2 and 3 were recovered from the gel, and each was analyzed further on a sequencing gel (Figure 6B) to identify the four sites of double-strand cleavage. Additional analysis of the DNAs in bands 2a and 3a of the nondenaturing gel permitted differentiation of the coupled double-strand cleavage events (A_{12}/C_{52} and C_{16}/C_{48}) from those double-strand cleavages that had resulted from closely spaced but independent single-strand cleavage events (T_{10}/A_{55} and C_{14}/C_{50}). A summary of the cleavage sites is shown in Figure 7, along with the previously determined patterns[10] for DNAs 7 and **10**, the latter of which involved double-strand cleavage at five and three sites, respectively. Of these 12 total sites, seven involved coupled double-strand cleavage while five resulted from closely spaced but independent cleavage events.

Double-Strand Cleavage of Methylated DNAs. The effect of methylation on double-strand DNA cleavage was assessed initially using hairpin DNA **10**, which was methylated at a single site (C_{13}) to afford DNA **10-Me**. As shown in Figure 8 for an experiment conducted under single-hit conditions [1 min treatment with 5 μM Fe(II)·BLM A_5], methylation of this DNA at C_{13} resulted in a significant reduction in the overall extent of double-strand cleavage of the hairpin DNA (20 to 11% overall double-strand cleavage for both end-labeled hairpin DNAs). There was also a large decrease in cleavage at the double-strand cleavage site closest to the site of methylation (C_{13}/G_{52}, 10 to 2% for the 5′-end-labeled DNA and 9 to 2% for the 3′-end-labeled DNA) with a large increase in double-strand

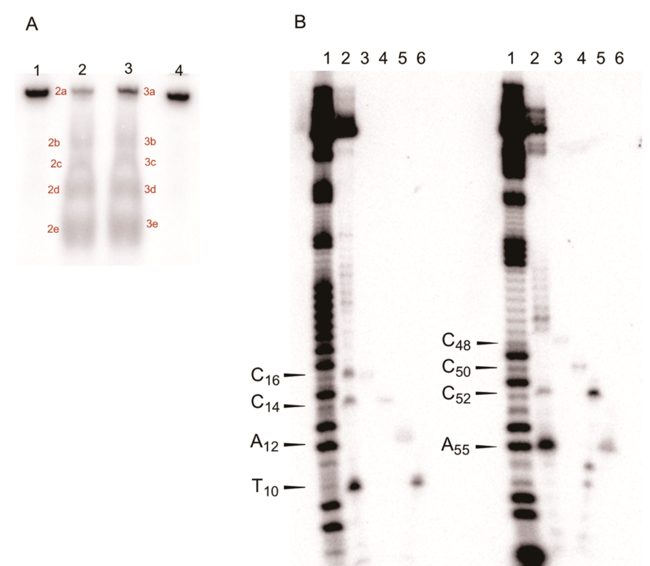

Figure 6. (A) Native gel electrophoresis of double-strand cleavage products of 5′-^{32}P-end-labeled (lane 2) and 3′-^{32}P-end-labeled (lane 3) 64-nucleotide hairpin DNA **11** by bleomycin A_5: lane 1, 5′-^{32}P-end-labeled DNA alone; lane 2, 5 μM Fe(II)·BLM A_5; lane 3, 5 μM Fe(II)·BLM A_5; lane 4, 3′-^{32}P-end-labeled DNA alone. (B) The left panel shows sequencing gel analysis of bleomycin-induced double-strand cleavage sites of 5′-^{32}P-end-labeled DNA **11** (lanes 1−6):[10] lane 1, Maxam−Gilbert G+A sequencing marker of 5′-^{32}P-end-labeled DNA **11**; lane 2, band 2a; lane 3, band 2b; lane 4, band 2c; lane 5, band 2d; lane 6, band 2e. The right panel shows sequencing gel analysis of bleomycin-induced double-strand cleavage sites of 3′-^{32}P-end-labeled DNA **11** (lanes 1−6): lane 1, Maxam−Gilbert G+A sequencing marker of 3′-^{32}P-end-labeled DNA **11**; lane 2, band 3a; lane 3, band 3b; lane 4, band 3c; lane 5, band 3d; lane 6, band 3e.

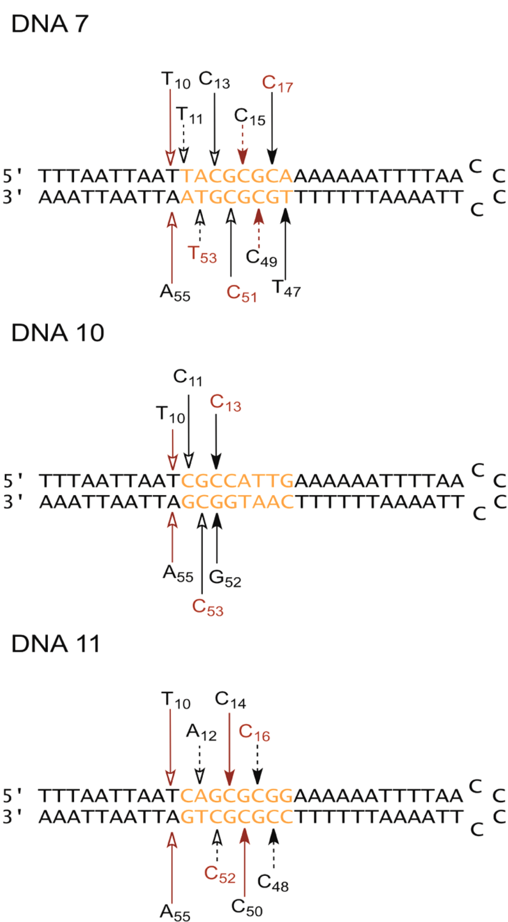

Figure 7. Summary of Fe·bleomycin-induced double-strand cleavage sites on DNAs **7**, **10**, and **11**. Orange bases indicate the randomized region of the original hairpin DNA library. Arrows of the same shape and color indicate paired cleavages. Black arrows correspond to coupled double-strand cleavage events, whereas red arrows indicate noncoupled double-strand cleavage events, resulting from two independent single-strand cleavages on opposite strands. Nucleotides colored red indicate primary sites of coupled double-strand DNA cleavage.

cleavage at the site most distant from the site of methylation (T_{10}/A_{55}, 2 to 6% for both end-labeled DNAs). Interestingly, when double-strand cleavage of DNAs **10** and **10-Me** was analyzed under more forcing conditions [30 min treatment with 5 μM Fe(II)·BLM A_5] intended to maximize double-strand cleavage, double-strand cleavage of DNA **10-Me** was also approximately one-half of that obtained using DNA **10**, and the relative intensities of double-strand cleavage were also shifted as in Figure 8 (Figure S2 of the Supporting Information). In this case, the large increase in cleavage at site C_{13}/G_{52} was counterbalanced by a relative decrease in cleavage at site C_{11}/C_{53} as well as T_{10}/A_{55}.

Also studied was the effect of adding more methylated cytidines to hairpin DNA **10** at known sites of double-strand cleavage. This included a hairpin DNA containing 5-methylcytidine at positions C_{13} and C_{53}, and another containing 5-methylcytidine at positions C_{11}, C_{13}, and C_{53}. As shown in Figure 9, under the same conditions used to cleave DNA **10** and DNA **10-Me** (Figure 8), much less cleavage of DNA **10-2Me** and DNA **10-3Me** was observed. As might have been anticipated on the basis of the results in Figure 9, the additional methylated cytidines further reduced the binding of Fe·BLM to the DNAs (Table 2), which undoubtedly contributed to the smaller amount of cleavage seen in Figure 9.

Binding of Methylated Hairpin DNAs by Fe·BLM. In an effort to understand the basis for the diminished and altered cleavage of DNA following methylation, we employed a competition assay developed previously to define the relative binding affinities of Fe·BLM for hairpin DNAs.[45] The assay is based on the Fe·bleomycin-induced cleavage of a profluorescent 16-nucleotide hairpin DNA that is cleaved stoichiometrically by Fe·BLM and contains a fluorescent nucleotide (Figure 2) at the site of cleavage.[46] The intact 16-nucleotide DNA does not fluoresce upon excitation because of quenching by nucleotides within the hairpin. However, when the DNA is cleaved by Fe·BLM, the fluorescent nucleobase is released and fluoresces upon being excited at 310 nm.[45] Admixture of an equivalent of a strongly bound 64-nucleotide hairpin DNA to the reaction mixture inhibits binding to the 16-nucleotide DNA and results in a proportionate decrease in fluorescence. As shown in Figure 10B and Table 2, hairpin DNA **10-Me** was much less inhibitory with respect to fluorophore release (20%) than DNA **10** (85%) even though DNA **10-Me** contains only a single methylated cytidine. Additional methylation of C_{53} (DNA **10-2Me**) or of C_{11} and C_{53} (DNA **10-3Me**), both of which are at sites of DNA cleavage (Figure 7), further diminished DNA binding (Figure S3 of the Supporting Information and Table 2) in parallel with the observed diminution of DNA cleavage (Figure 9). Likewise, methylation of DNA **11** (at five cytidines) also significantly reduced its

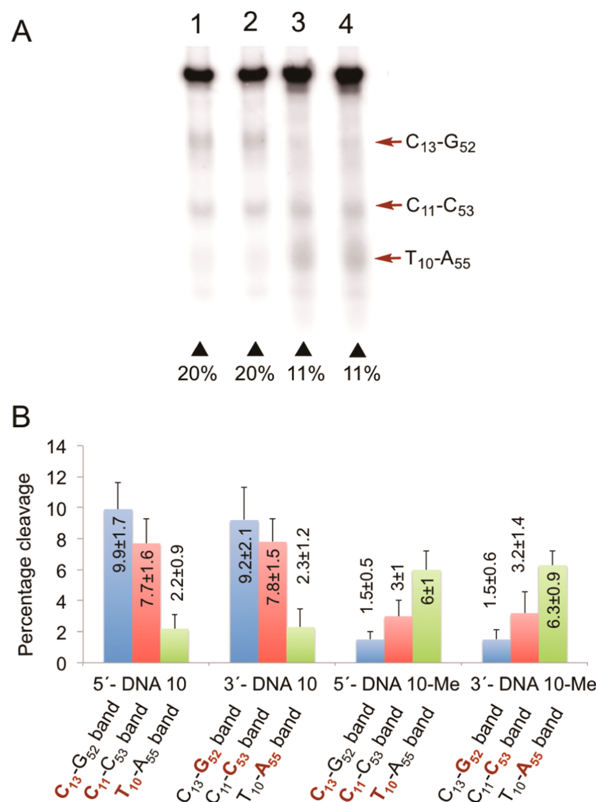

Figure 8. (A) Native polyacrylamide gel showing bands resulting from double-strand cleavage of 5′-^{32}P-end-labeled (lanes 1 and 3) and 3′-^{32}P-end-labeled (lanes 2 and 4) 64-nucleotide hairpin DNA **10** (lanes 1 and 2) and DNA **10-Me** (lanes 3 and 4) by 5 μM Fe(II)·BLM A_5 for 1 min. (B) Histogram illustrating a shift of double-strand DNA cleavage intensity from the C_{13}/G_{52} site to the neighboring sites upon methylation of C_{13}.

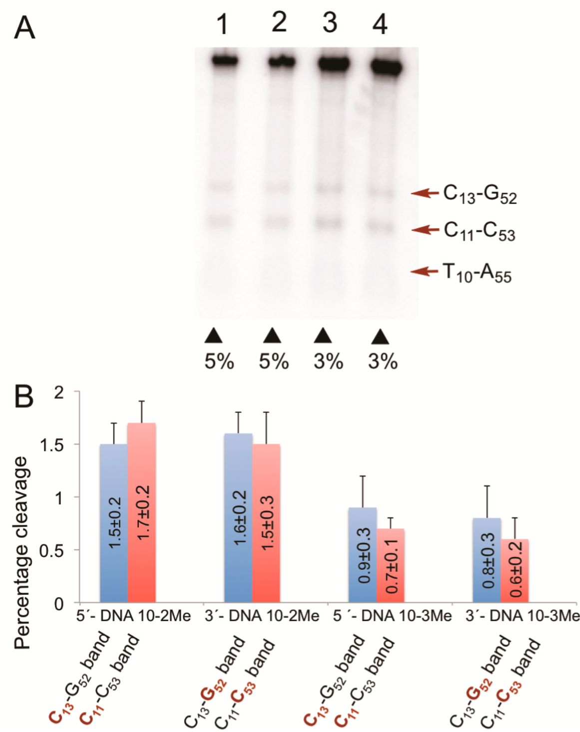

Figure 9. (A) Native polyacrylamide gel showing bands resulting from double-strand cleavage of 5′-^{32}P-end-labeled (lanes 1 and 3) and 3′-^{32}P-end-labeled (lanes 2 and 4) 64-nucleotide hairpin DNA **10-2Me** (lanes 1 and 2) and DNA **10-3Me** (lanes 3 and 4) by 5 μM Fe(II)·BLM A_5 for 1 min. (B) Histogram illustrating the overall decrease in double-strand DNA cleavage intensity of C_{13}/G_{52} and C_{11}/G_{53} sites upon methylation of C_{53} (DNA **10-2Me**) and C_{11} and C_{53} (DNA **10-3Me**) in addition to C_{13}.

ability to block the Fe·BLM-mediated degradation of the 16-nucleotide reporter DNA (Figure 10C and Table 2).

In comparison, DNAs **7** and **7-4Me** both substantially inhibited cleavage of the 16-nucleotide DNA. The difference seemed likely to be due to the presence of the unmethylated 5′-GC_{17} and 5′-GT_{53} sequences in DNA **7-4Me**, both of which are known primary sites of double-strand cleavage in DNA **7** (Figure 7).[10] To test this hypothesis, we investigated a related hairpin DNA in which $cytidine_{17}$ was also methylated (DNA **7-5Me**). As shown in Figure S4 of the Supporting Information and Table 2, this did reduce the ability of the hairpin DNA to compete with the profluorescent 16-nucleotide hairpin DNA (64% inhibition).

Structural Basis for Diminished Binding and Cleavage of DNAs Containing Methylated Cytidines. The introduction of a methyl substituent at position 5 of cytidine has little effect on the pK_a of the nucleobase[47] and introduces the substituent into the major groove of DNA while BLM conducts its chemistry in the minor groove.[32−34] Further, DNA already contains a methyl substituent at the same position of each deoxythymidine residue in DNA, and 5′-GT sites are often excellent sites for cleavage by Fe·BLM. Nonetheless, this does not exclude the possibility that in the absence of these methyl groups, (demethylated) DNA might be a better substrate for cleavage by Fe·BLM. Accordingly, we studied an analogue of hairpin DNA **9**, which has two 5′-GT sequences and utilizes both as primary sites for Fe·BLM-mediated double-strand cleavage.[10] Both deoxythymidine residues were replaced with deoxyuridine, and the modified DNA **9-2dU** was employed as a substrate for Fe(II)·BLM. As shown in Table 1, DNA **9-2dU** was cleaved to a slightly greater extent (27%) than DNA **9** (20%), in parallel with its tighter binding by Fe(II)·BLM [95 vs 90% binding specificity (Figure S5 of the Supporting Information and Table 2)].

To better define the effect of the methyl groups normally found on dT residues in DNA on Fe·BLM-mediated DNA cleavage, we also compared the cleavage of DNA **9** and DNA **9-**

Table 2. Inhibition of Fluorescence Emission by Hairpin DNAs[a]

hairpin DNA	binding specificity (%)
DNA 7	97 ± 1
DNA **7-4Me**	91 ± 2
DNA **7-5Me**	64 ± 4
DNA **9**	90 ± 2
DNA **9-2dU**	95 ± 1
DNA **10**	85 ± 1
DNA **10-Me**	20 ± 3
DNA **10-2Me**	14 ± 2
DNA **10-3Me**	11 ± 1
DNA **11**	97 ± 1
DNA **11-5Me**	13 ± 3

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

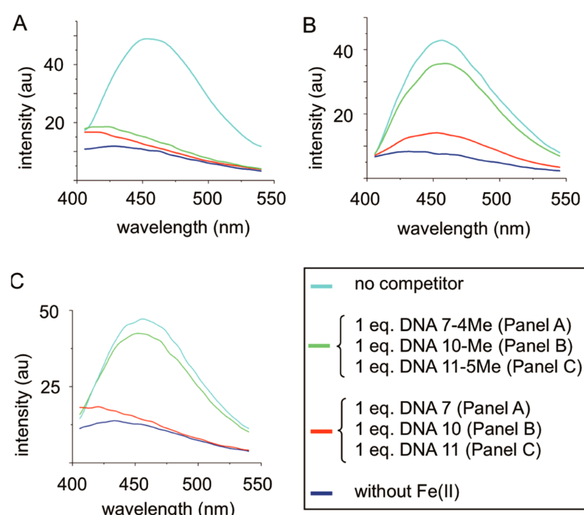

Figure 10. Fluorescence emission spectra obtained following treatment of the profluorescent 16-nucleotide hairpin DNA C_{f15} (Figure 2) with Fe(II)·BLM in the presence or absence of 1 equiv of a 64-nucleotide hairpin DNA. (A) Emission spectra obtained following treatment of 1 μM 16-nucleotide hairpin DNA with 1 μM Fe(II)·BLM A_5 with or without 1 μM DNA **7** or DNA **7-4Me**. The emission of 1 μM 16-nucleotide hairpin DNA following treatment with metal-free BLM A_5 was used as a control. The emission spectra were obtained following excitation at 310 nm. (B) Emission spectra obtained following treatment of 1 μM 16-nucleotide hairpin DNA with 1 μM Fe(II)·BLM A_5 with or without 1 μM DNA **10** or DNA **10-Me**. The emission of 1 μM 16-nucleotide hairpin DNA following treatment with metal-free BLM A_5 was used as a control. (C) Emission spectra obtained following treatment of 1 μM 16-nucleotide hairpin DNA with 1 μM Fe(II)·BLM A_5 with or without 1 μM DNA **11** or DNA **11-5Me**. The emission of 1 μM 16-nucleotide hairpin DNA following treatment with metal-free BLM A_5 was used as a control.

2dU at lower temperatures, where Fe·BLM cleaves B-form DNA less quickly, presumably reflecting the presence of a more compact DNA structure. As shown in Figure 11, under these conditions, DNA **9-2dU** was cleaved to a greater extent (2−3-fold) after 30 min than was DNA **9**. Thus, methylation of DNA in the major groove at deoxyuridine or deoxycytidine residues diminishes both DNA binding and cleavage, the latter of which involves minor groove chemistry.

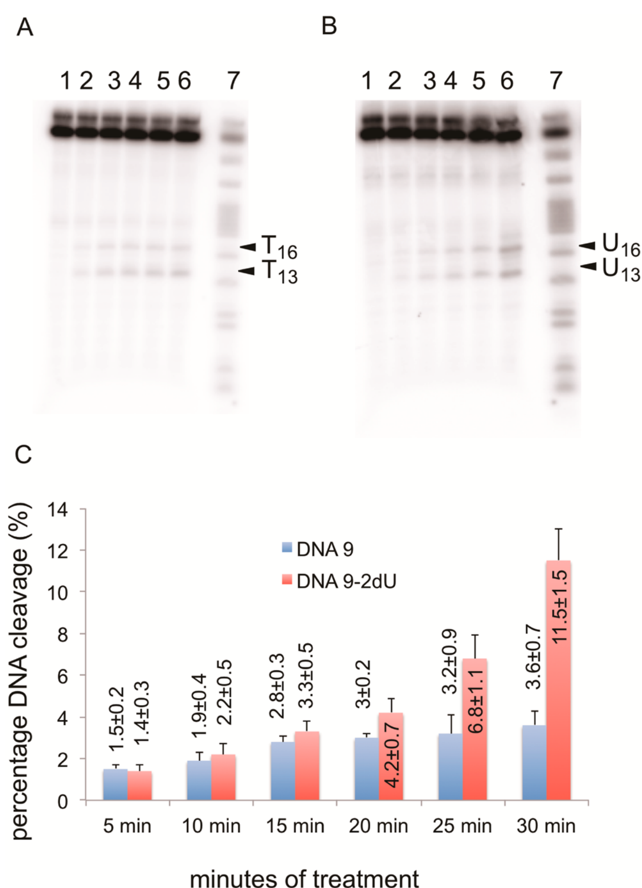

Figure 11. (A) Time-dependent, sequence selective cleavage of 5′-^{32}P-end-labeled DNA **9** by 5 μM Fe(II)·BLM A_5 at 0 °C: lane 1, 5 min; lane 2, 10 min; lane 3, 15 min; lane 4, 20 min; lane 5, 25 min; lane 6, 30 min; lane 7, G+A lane. (B) Time-dependent, sequence selective cleavage of 5′-^{32}P-end-labeled DNA **9-2dU** by 5 μM Fe(II)·BLM A_5 at 0 °C: lane 1, 5 min; lane 2, 10 min; lane 3, 15 min; lane 4, 20 min; lane 5, 25 min; lane 6, 30 min; lane 7, G+A lane. (C) Histogram representing time-dependent enhanced cleavage of DNA **9-2dU**.

DISCUSSION

Methylated DNAs as Substrates for Bleomycin Binding and Cleavage. Although two early reports from our laboratory noted effects of DNA methylation on DNA cleavage by bleomycin,[20,21] no systematic study of the effects of DNA methylation on DNA binding or cleavage has been reported. Given the presence of thymidine (5′-deoxyuridine) as one of the four canonical nucleobases in DNA, and the highly efficient cleavage of many 5′-GT sequences in B-form DNA by Fe(II)·BLM, a consideration of the possible effects of pyrimidine nucleobase methylation might be considered illogical. Nonetheless, our recent focus on factors that render some DNA structures amenable to high-affinity binding by bleomycin under conditions where the drug is limiting, such as in a therapeutic setting, suggested the need to consider the effects of naturally occurring DNA modifications. DNA cytidine methylation has been shown to affect nucleosome structure[48] and gene expression and is altered in many cancers, again arguing for the need to consider DNA methylation as one possible factor influencing the action of bleomycin as a therapeutic agent.

In several recent studies, we have provided evidence that suggests that DNAs having a strong affinity for Fe·BLM are likely to be bound selectively when numerous DNAs having varying affinities for Fe·BLM are targeted by a limiting amount of the drug.[10,22,45,49−51] Further, such DNAs undergo a newly recognized type of double-strand DNA cleavage that could lead to the production of double-strand gaps in duplex DNA[10] and contribute to cell killing. Given the demonstrated selective targeting of tumor cells by BLM,[52−54] the creation of double-strand gaps in certain DNAs within tumor cells may be relevant to the mechanism of action of BLM as an antitumor agent. While there are numerous sites of DNA methylation within the human genome in both normal and cancer cells, considering the effect of DNA methylation in DNAs known to be bound strongly by Fe·BLM in the absence of cytidine methylation may plausibly provide additional insights into characteristics of DNAs that are the targets responsible for tumor cell killing by bleomycin.

Effects of DNA Methylation on Binding and Cleavage by Fe·BLM. For the strongly bound hairpin DNAs utilized in this study, not all cytidine methylations had the same impact on bleomycin binding. Thus, methylation at a single position (C_{13})

in DNA **10** substantially diminished binding of Fe(II)·BLM A_5, and this effect was not greatly enhanced by the introduction of additional methylation at C_{53} (Table 2). This bis-methylated species (DNA **10-2Me**) had the same BLM binding specificity as DNA **11-5Me**, containing five methylated cytidines (Table 2). In contrast, the methylation at four sites in DNA **7** (DNA **7-4Me**) had little effect on the binding specificity of that hairpin DNA, in spite of the fact that DNA **7** and DNA **11** themselves exhibited identical affinities for Fe(II)·BLM A_5 (Table 2). The more substantial effect of adding a fifth methyl group to DNA **7-4Me** (affording DNA **7-5Me**) at a known site of double-strand DNA cleavage [C_{17} (Figure S4 of the Supporting Information and Table 2)] suggests that the site of methylation is important to its effect on Fe·BLM binding.

The same conclusion can be reached by considering the effects of DNA cytidine methylation on Fe·BLM-mediated DNA cleavage. As shown in Figure 8 and Figure S2 of the Supporting Information for DNA **10**, methylation at C_{13} results both in an overall diminution of DNA cleavage (Table 1) and in a shift of the observed cleavage to sites distant from the site of methylation. This implies that methylation may shift the preferred site(s) of DNA binding. The same effect was also apparent in Figures 3 and 4 upon methylation of DNA **7**.

Molecular Basis for the Effects of Major Groove Modifications on the Binding and Cleavage of DNAs by Bleomycin. There are numerous examples of the selective cleavage of BLM involving specific sequences and higher-order structures in both DNA and RNA. When excess Fe(II)·BLM is employed to cleave B-form DNAs, the major sites of cleavage involve 5′-GT and 5′-GC sequences,[6,7,32−34] and 5′-GPy sequences also figure prominently as primary sites of cleavage in coupled double-strand DNA cleavage events.[9] It is believed that DNA binding by Fe·BLM must precede cleavage, limiting cleavage to a subset of those DNA and RNA sites bound effectively by Fe·BLM, and having the bound Fe·BLM−DNA complex in an orientation that permits abstraction of a H atom from C-4′ of (deoxy)ribose in the rate-limiting chemical step.[55]

The minor groove of B-form DNA is relatively wide and shallow at 5′-GT and 5′-GC sequences, and this feature could plausibly favor binding to Fe·BLM. Consistent with this thesis, Fe·BLM was found to cleave the duplex domain of a DNA triplex at the 5′-duplex−triplex junction, a site suggested by computational modeling to have a minor groove somewhat wider and shallower than that in canonical B-form DNA.[56] Likewise, the cleavage of tRNAs by Fe·BLM occurred disproportionately at the junction between single- and double-strand regions, where the minor groove-like structures might be expected to be relatively wide,[57] and proved to be very sensitive to even small changes in tRNA structure.[58]

As noted in Tables 1 and 2, the methylation of cytidine nucleobases in the major groove of hairpin DNAs **7**, **10**, and **11** led in each case to decreased Fe·BLM binding affinity and a decrease in DNA cleavage products. Because the chemistry of DNA cleavage involves initial abstraction of a H atom from the C-4′ position of deoxyribose in the DNA minor groove, the diminution of DNA binding and cleavage could not have resulted from a direct steric interaction between the methyl group of 5-methylcytidine and Fe·BLM. However, it seemed possible that introduction of one or more methyl groups within the DNA major groove might result in steric interactions that were relieved in part by compressing the DNA minor groove, thereby affecting Fe·BLM binding and cleavage. It is also possible that the reported decrease in flexibility that accompanies DNA methylation[59−61] may have an effect on DNA binding and cleavage by Fe·BLM.

If the introduction of methyl groups into the DNA major groove was the source of diminished Fe·BLM-mediated cleavage in the DNA minor groove, then the removal of such methyl groups might be expected to increase Fe·BLM binding and cleavage. In fact, the 10 hairpin DNAs in the library studied to date all had thymidine residues within the 8 bp region originally randomized to produce the library. Thus, all of the DNA binding and cleavage studies have been conducted in a background of (per)methylated major grooves. Hairpin DNA **9** has five thymidine residues, two of which (T_{13} and T_{16}) are primary sites of double-strand cleavage by Fe·BLM.[10] Replacement of these two thymidines with deoxyuridine residues resulted in an increased binding affinity for Fe·BLM (Table 2) and increased DNA cleavage products (Table 1). While the differences were not large, when hairpin DNAs **9** and **9-2dU** were compared at 0 °C, where DNA cleavage by Fe·BLM is intrinsically slower, a 2−3-fold increase in cleavage was noted for DNA **9-2dU** after 25−30 min.

Modulation of minor groove width has been documented in a number of systems, including DNAs that are intrinsically bent,[62] as a consequence of protein binding to the DNA major groove,[63,64] and to accommodate the parallel binding of hairpin polyamide DNA binders.[65] The results presented here suggest that Fe·BLM is sufficiently sensitive to the dimensions of the DNA minor groove to recognize changes in geometry caused by the introduction of methyl groups into pyrimidine nucleobases in the DNA major groove.

■ CONCLUSIONS

In conclusion, we have demonstrated that cytidine methylation in DNA reduces the overall cleavage mediated by Fe·bleomycin and also changes the facility and pattern of double-strand DNA cleavage. Cytidines, being part of highly conserved CpG sequences, have direct involvement in epigenetic gene regulation, developmental processes, and carcinogenesis.[66,67] The nonmethylated and methylated hairpin DNAs employed in this study have provided a model system that has permitted the analysis of the effects of cytidine methylation on single- and double-strand DNA breaks mediated by Fe·BLM. To the extent that this study is predictive of events in more complex cellular systems, it suggests that DNA methylation in normal cells could protect them from the cytotoxic effects of the antitumor agent bleomycin, while focusing the effects of the drug on the (undermethylated) DNA in cancer cells. The dramatic decrease in binding of Fe·BLM to the methylated cytidines in the proximity of BLM cleavage sites in our model hairpin DNAs may plausibly contribute to the selective action of BLM toward the DNA in tumors as compared with normal cells in their response to treatment with bleomycin, especially given that the extent of double-strand DNA cleavage, the putative lethal event mediated by BLM, has recently been found to correlate with DNA binding affinity.[10]

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental procedures for the DNA radiolabeling, cleavage, and competition assay. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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