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Hairpin DNA Sequences Bound Strongly by Bleomycin Exhibit Enhanced Double-Strand Cleavage

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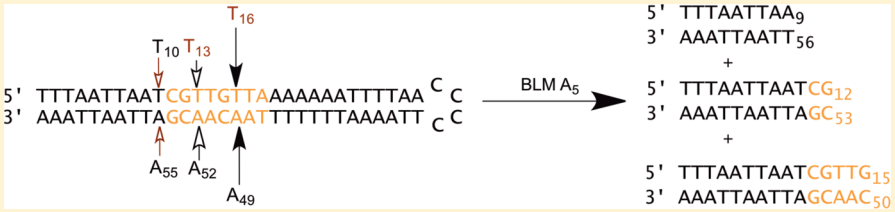
Supporting Information



ABSTRACT: Clinically used bleomycin A_5 has been employed in a study of double-strand cleavage of a library of 10 hairpin DNAs originally selected on the basis of their strong binding to bleomycin. Each of the DNAs underwent double-strand cleavage at more than one site, and all of the cleavage sites were within, or in close proximity to, an eight-base-pair region of the duplex that had been randomized to create the original library. A total of 31 double-strand cleavage sites were identified on the 10 DNAs, and 14 of these sites were found to represent coupled cleavage sites, that is, events in which one of the two strands was always cleaved first, followed by the associated site on the opposite strand. Most of these coupled sites underwent cleavage by a mechanism described previously by the Povirk laboratory and afforded cleavage patterns entirely analogous to those reported. However, at least one coupled cleavage event was noted that did not conform to the pattern of those described previously. More surprisingly, 17 double-strand cleavages were found not to result from coupled double-strand cleavage, and we posit that these cleavages resulted from a new mechanism not previously described. Enhanced double-strand cleavages at these sites appear to be a consequence of the dynamic nature of the interaction of Fe·BLM A_5 with the strongly bound hairpin DNAs.

INTRODUCTION

The antitumor agent bleomycin is the prototypic sequence-selective DNA cleaving agent. That property has been studied intensively for decades in the belief that it must form the basis for the antitumor activity of bleomycin (BLM).[1−4] Of special interest was the finding of double-strand DNA cleavage at a frequency greatly in excess of what could result from the random accumulation of single-strand breaks.[5,6] Nonetheless, the modest potency of bleomycin as a DNA cleaving agent and the repair of BLM-mediated DNA damage[7] have complicated efforts to define its cytotoxic mechanism.

One impediment to elucidating the BLM−DNA interactions critical to the expression of antitumor activity has been the routine use of an excess of BLM relative to DNA in cell-free studies of DNA cleavage. Given the low (∼5 μmol) clinical dose of BLM, it must certainly be the case that chromosomal DNA is in excess in a therapeutic setting. Since DNA binding precedes cleavage by BLM[1−4,8] and only a limited turnover of BLM in DNA cleavage has ever been documented,[9,10] under such circumstances, only those DNAs bound avidly by BLM would have any chance of being cleaved by the drug.

Recent studies have utilized a library of hairpin DNAs to permit the selection of DNAs that bind tightly to BLM (Table 1).[11,12] A number of those strongly bound DNAs were used as substrates to define the dynamics of BLM binding to DNA[13] and DNA cleavage patterns.[12,14,15] Two key findings were that (i) the DNAs studied had only a single site at which BLM bound strongly, but were nonetheless cleaved at numerous positions; and (ii) even when the BLMs were not bound to the DNAs as judged by surface plasmon resonance, the BLMs were unavailable to cleave less strongly bound DNAs.[13]

Presently, we analyze the double-strand cleavage of 10 hairpin DNAs bound strongly by BLM. All 10 DNAs undergo multiple, closely spaced, double-strand DNA cleavage events, and some of these involve a novel mechanism. If operative in a therapeutic setting, these processes could result in the creation of double-strand gaps in duplex DNA, a process that could well account for the observed cytotoxic activity of BLM.

RESULTS

We have recently reported the positions of cleavage of 10 hairpin DNAs that bind BLM strongly (Supporting Information (SI) Figure S1).[15] To determine whether any of these cleavage events involved double-strand cleavage, a few DNAs with modest numbers of cleavage sites in proximity to each other on opposite strands of the formed duplex were chosen; these included hairpin DNAs **2**, **8**, and **9** (SI Figure S1). Each of these DNAs was alternatively ^{32}P end-labeled at the 5′ or 3′-end and then treated individually with Fe(II)·BLM A_5 (Figure 1). As shown for DNA

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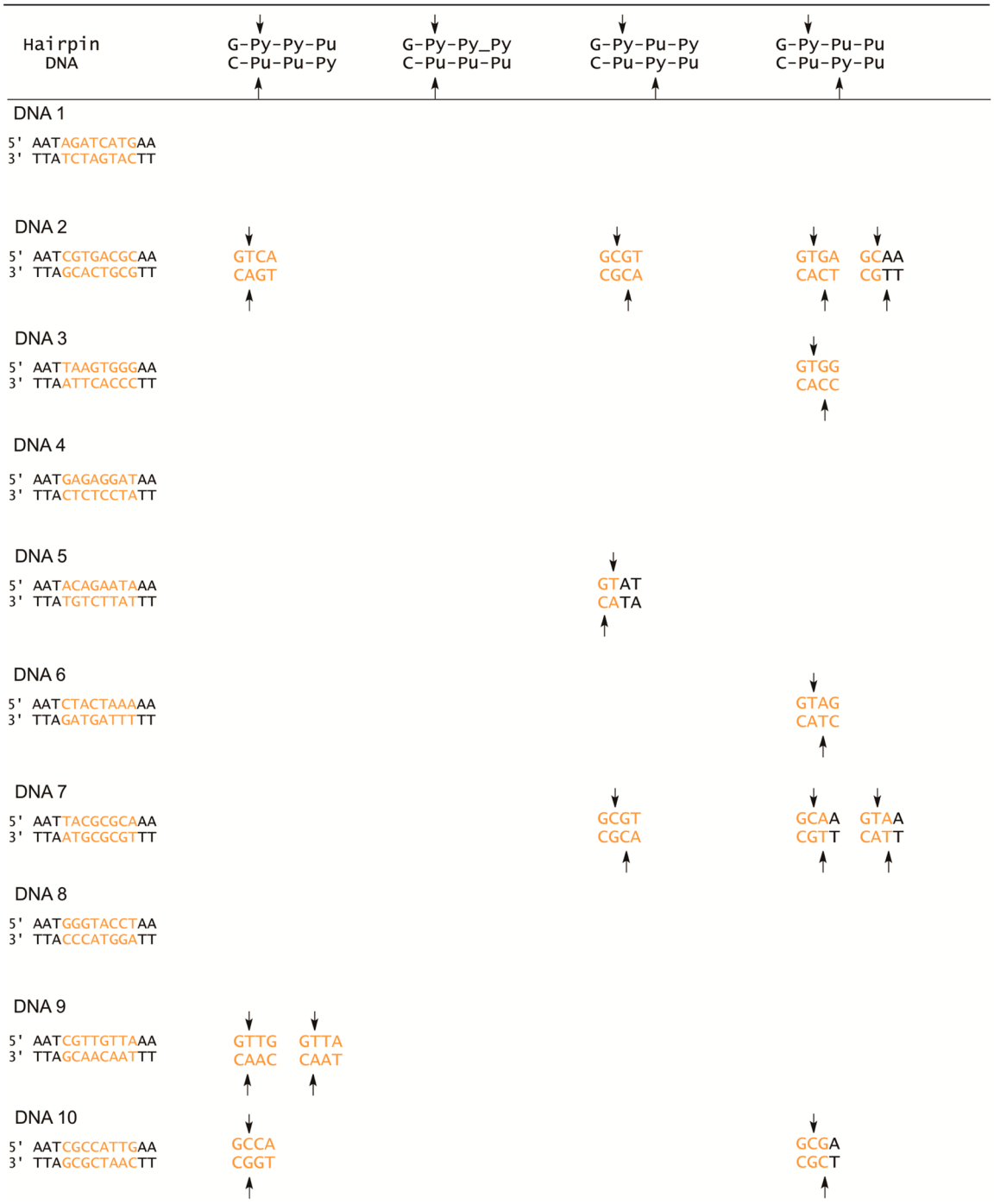
Table 1. Coupled Double-Strand BLM Cleavage Sites in Hairpin DNAs 1−10[a]



[a]Cleavage sequences aligned with primary G-Py cleavage sites.

8 in Figure 2, three bands labeled on opposite ends of the DNA comigrated in the nondenaturing gel.[16] These must represent double-strand cleavage events, affording short duplexes radiolabeled at the 5′ or 3′-end. Bands were also apparent in each lane that comigrated with the intact hairpin DNA.

The chemistry of DNA strand scission by Fe(II)·BLM has been studied extensively and shown to involve two processes, both involving initial abstraction of the C-4′ H atom of susceptible DNA sugars by the activated form of Fe·BLM.[1−4,8,17,18] One process results in frank strand scission of DNA, affording DNA fragments having 3′-phosphoroglycolate and 5′-phosphate termini, with the release of the oxidized nucleoside as a nucleobase propenal.[9,17−23] The other process, which is enhanced at lower oxygen tension, affords no DNA strand scission, but results in the release of the free nucleobase from the oxidized nucleoside with the concomitant formation of a C-4′ hydroxylated apurinic (apyrimidinic) acid.[2,17−26] This lesion is chemically labile; DNA strands containing this lesion can be cleaved with reagents such as alkali, an alkylamine such as *n*-butylamine, or hydrazine.[26,27]

The double-strand hairpin DNA cleavage products shown in Figure 2 must, therefore, arise as summarized in Figure 3. As shown, the initial BLM-induced lesion can involve the formation

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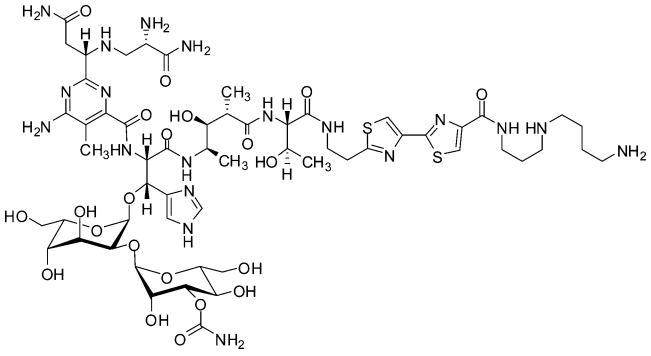

Figure 1. Chemical structure of bleomycin A_5.

of an alkali-labile lesion (I), which will not lead directly to strand scission, or to the strand scission process affording the nicked DNA duplex II, as described above. The latter can undergo a second Fe·BLM-mediated oxidation reaction in proximity to the first lesion, but on the opposite strand, to afford either an alkali-labile lesion on the second strand (product III) or frank strand scission (product IV). Product IV is the only one involving double-strand cleavage, but treatment of III with alkali, *n*-butylamine, or hydrazine would also be expected to lead to a related double-strand cleavage product (V). In fact, as shown in Figure 2, the initially formed double-strand DNA cleavage products (b, c, and d in lanes 2 and 4) were all enhanced in amount following additional treatment of the reaction mixtures with *n*-butylamine (affording products b, c, and d in lanes 3 and 5), whereas the bands corresponding to full length hairpin DNA (a in lanes 2 and 4) were diminished in intensity. As noted in Figure 2, the species that comigrate with full length hairpin DNA are attributed to products of type I, II, and III and possibly to residual amounts of the intact hairpin DNA. All of the cleaved products (b, c, and d) formed prior to *n*-butylamine treatment are believed to be of type IV.

The positions of cleavage of products b, c, and d in Figure 2 were assigned by isolating each from the original gel and subjecting each to denaturing 20% polyacrylamide gel electrophoresis in direct comparison with a Maxam–Gilbert G+A sequencing lane. As shown in Figure 4, the [3′-^{32}P]-end-labeled product 4b comigrated with the band in the sequencing lane corresponding to cleavage at T_{50}. Product 5b (additionally treated with *n*-butylamine) was also cleaved at T_{50}. Likewise, 4c and 4d were found to have been cleaved at positions C_{52} and T_{56}, respectively. As shown in lane 2 of Figure 4, product 4a gave bands that comigrated with each of the three products derived from 4b, 4c, and 4d. This indicated that the duplex in 4a must have contained products having undergone strand scission at positions T_{50}, C_{52}, and T_{56}, but with no cleavage on the opposite strand, that is, of type II or III. Thus, all three of these sites can represent primary sites of cleavage (cf Figure 3).

The same process was employed to identify the sites of cleavage of the [5′-^{32}P]-end-labeled products derived from hairpin DNA **8**. As shown in lanes 9, 11, and 12 of Figure 4, the products in bands 2b, 2c, and 2d were cleaved at positions T_{14}, G_{12}, and A_9, respectively. Thus, the double-strand cleavage product in band b (Figure 2) must have resulted from cleavage at T_{14} and T_{50}, the product in band c must have resulted from cleavage at G_{12} and C_{52}, and the product in band d resulted from cleavage at A_9 and T_{56}. The sequences of the three double-strand cleavage products are summarized in Figure 5. As shown in lane 8 of Figure 4, band 2a (from Figure 2) gave bands at positions A_9, G_{12}, and T_{14} when run

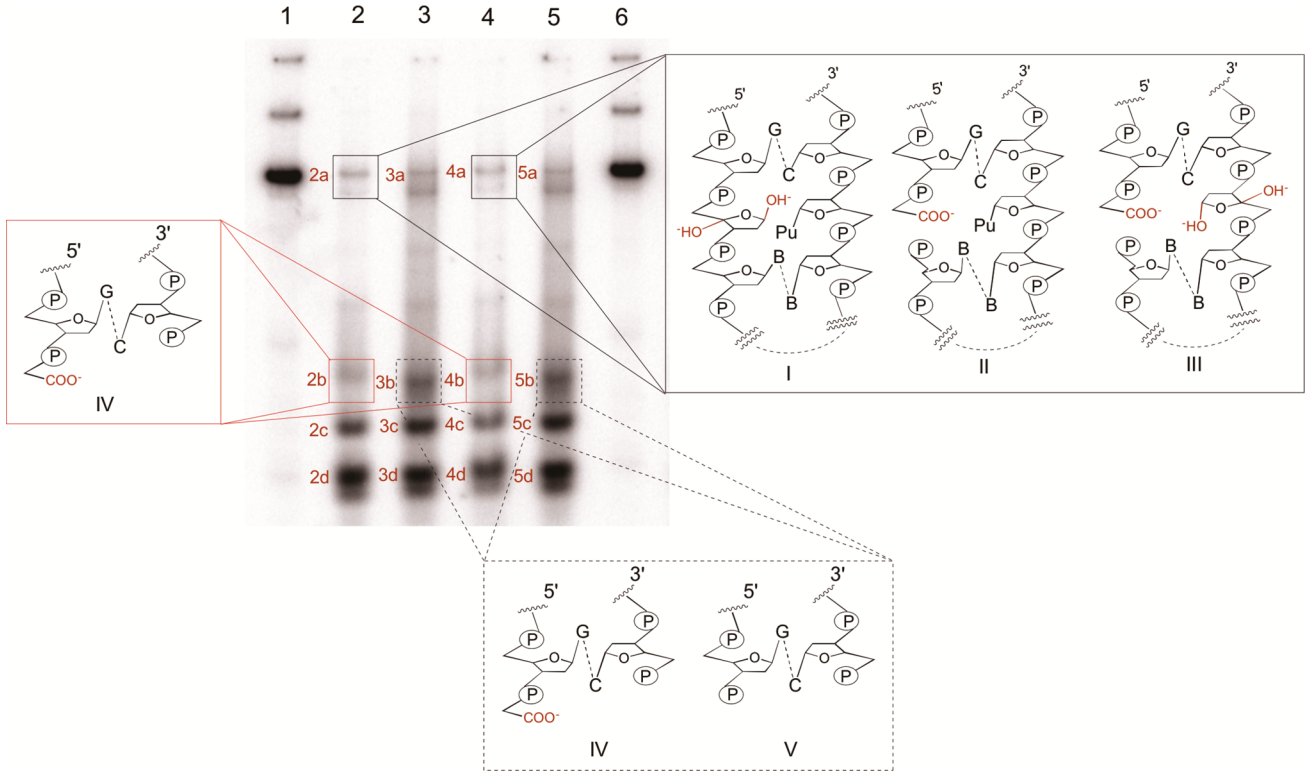

Figure 2. Double-strand cleavage of [5′-^{32}P]-end-labeled (lanes 1–3) and [3′-^{32}P]-end-labeled (lanes 4–6) 64-nucleotide hairpin DNA **8** by Fe(II)·BLM A_5. Lane 1, [5′-^{32}P]-end-labeled DNA alone; lane 2, 5 μM Fe(II)·BLM A_5; lane 3, 5 μM Fe(II)·BLM A_5 + 2 mM *n*-butylamine; lane 4, 5 μM Fe(II)·BLM A_5; lane 5, 5 μM Fe(II)·BLM A_5 + 2 mM *n*-butylamine; lane 6, [3′-^{32}P]-end-labeled DNA alone.

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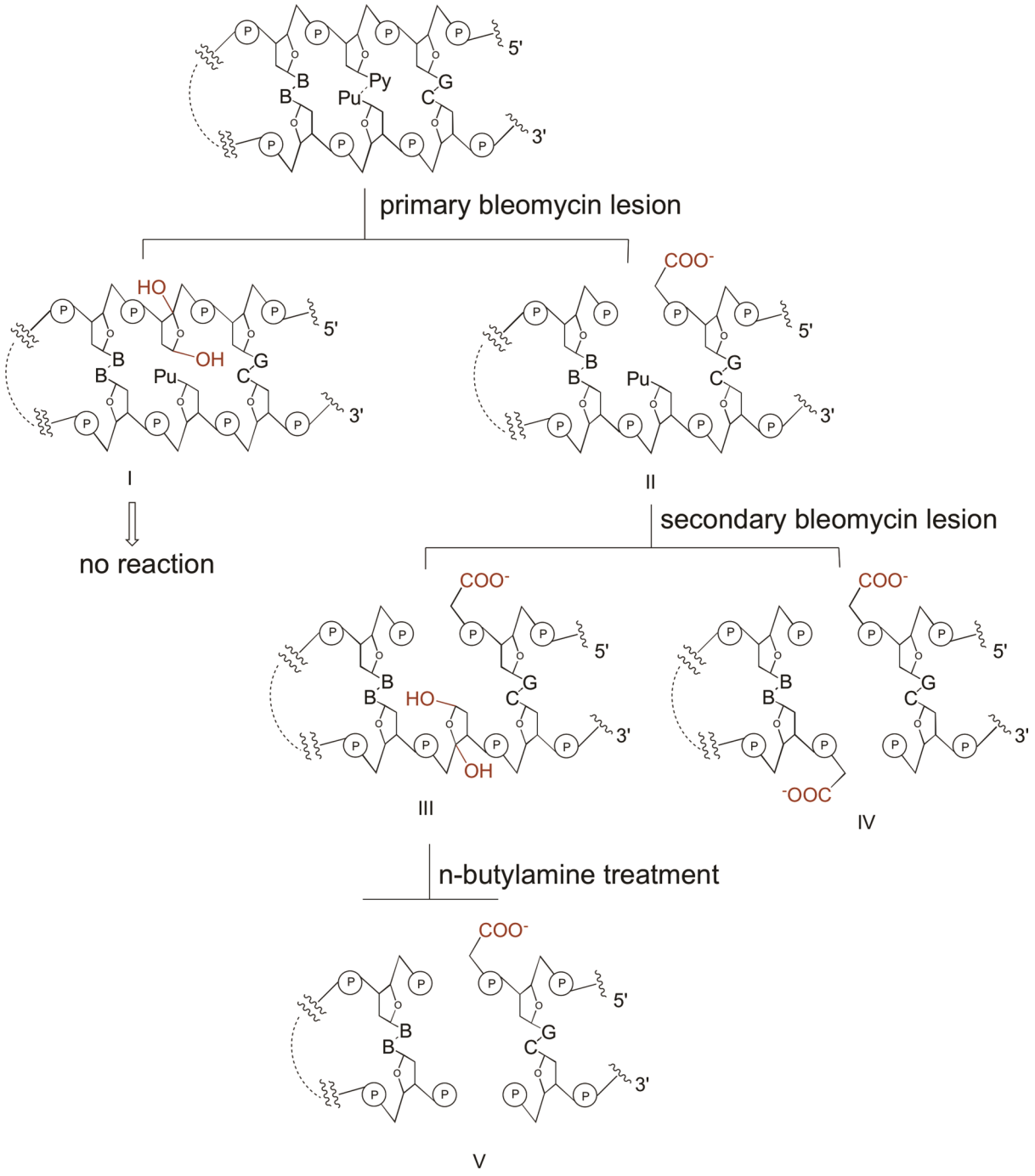

Figure 3. Mechanisms of bleomycin induced double-strand DNA cleavage. Activated Fe·BLM abstracts a H atom from the C-4′position of deoxyribose at the primary site (typically 5′-G-**Py**-B-3′ of one strand of the hairpin DNA, where B = any nucleobase), producing either an AP (apyrimidinic/apurinic) site (I) or a single-strand break terminating with a 3′-phosphoroglycolate (II). Although 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/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 (V).

on the denaturing polyacrylamide gel. Thus, each of these positions was cleaved in some of the hairpin DNAs, but with no cleavage at proximal sites on the opposite strand. Thus, each of these sites can also be primary sites of cleavage (cf Figure 3). This indicates the absence of evidence for any obligatory order of cleavage for the three pairs of double-strand cleavage sites on DNA **8**.

Shown in Figure 6 is the native gel analysis of the cleavage products resulting from treatment of hairpin DNA **2** with Fe(II)·BLM A_5. Five new bands are apparent in addition to one (a) that comigrated with intact hairpin DNA **2**. Further, the five bands in the [5′-^{32}P]- and [3′-^{32}P]-end-labeled DNAs comigrated, consistent with double-strand cleavage.[16,28] Each of the six bands in

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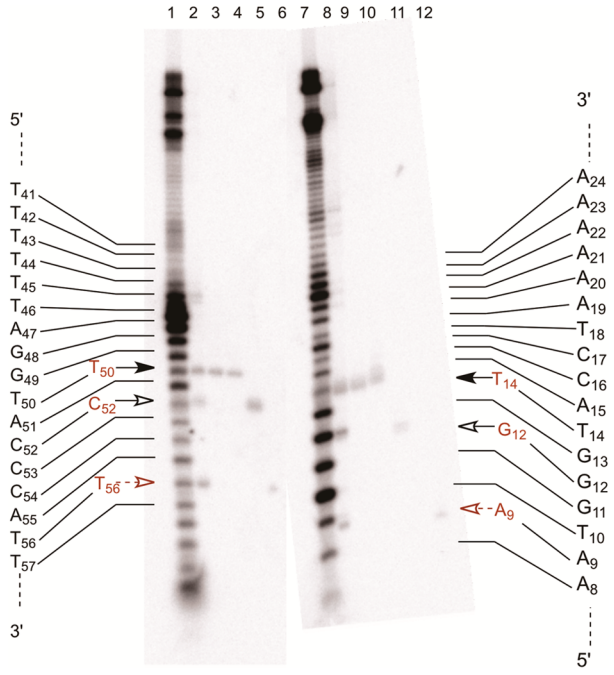

Figure 4. Sequencing gel analysis of Fe(II)·BLM A_5-induced double-strand cleavage sites of [3′-^{32}P]-end-labeled (lanes 1−6) and [5′-^{32}P]-end-labeled (lanes 7−12) DNA **8**. Each lane (except lanes 1 and 7) corresponds to a numbered cleavage band, shown in Figure 2. Lane 1, Maxam−Gilbert G+A sequencing lane of [3′-^{32}P]-end-labeled DNA **8**; lane 2, band 4a; lane 3, band 4b; lane 4, band 5b; lane 5, band 4c; lane 6, band 4d; lane 7, Maxam−Gilbert G+A sequencing lane of [5′-^{32}P]-end-labeled DNA **8**; lane 8, band 2a; lane 9, band 2b; lane 10, band 3b; lane 11, band 2c; lane 12, band 2d.

lanes 2 and 3 was isolated and subjected to analysis by denaturing polyacrylamide gel electrophoresis.

As shown in Figure 7, the [5′-^{32}P]-end-labeled DNA **2** was cleaved at positions A_9, T_{13}, A_{15}, C_{16}, and C_{18}, whereas the [3′-^{32}P]-end-labeled DNA **2** was cleaved at positions T_{46}, C_{48}, T_{50}, C_{51}, and T_{56}.

Co-migrating bands 2b and 3b (Figure 6) contained DNAs cleaved at positions T_{46} and C_{18}, respectively, indicating that these were the cleavage sites leading to the formation of this double-strand cleavage product. Co-migrating bands 2c and 3c (Figure 6) were found to contain DNAs cleaved at C_{48} and C_{16}, respectively. Comparable analysis of the other comigrating bands in Figure 6 (2d/3d, 2e/3e, and 2f/3f) indicated that double-strand cleavage of these DNAs involved cleavage at T_{50}/A_{15}, C_{51}/T_{13} and T_{56}/A_9, respectively (Figure 7). The sequences of the five products of double-strand cleavage are summarized in Figure 5.

Another facet of the cleavage of hairpin DNA **2** became apparent when bands 2a and 3a (Figure 6) were analyzed by denaturing polyacrylamide gel electrophoresis (Figure 7). The analysis of the product in band 2a (lane 7) indicated cleavage at positions C_{48} and T_{50}. In comparison, analysis of the product in band 3a (lane 14) indicated cleavage at positions T_{13} and C_{18}. As noted above, these must represent sites at which the duplexes in bands 2a and 3a had been nicked to give intermediates of type II or III (Figure 3) with no cleavage on the opposite strand. Each of these sites thus represents a primary cleavage site. Cleavages at T_{56} and A_9 were readily apparent in lanes 2 and 9, respectively, but not in lanes 7 and 14, respectively. This presumably reflects efficient cleavage at these sites to give bands 2f/3f, leaving little partially cleaved material in bands 2a/3a. Thus, we believe that cleavages at A_9 and T_{56} represent independent events. In comparison, the other four double-strand cleavage sites (T_{13}/C_{51}, A_{15}/T_{50}, C_{16}/C_{48}, and C_{18}/T_{46}) appear to have obligatory primary and secondary cleavage sites, consistent with a mechanism described previously.[28]

A third example of double-strand cleavage is provided for hairpin DNA **9** (Figure 8). As shown in Figure 8A, three double-strand cleavage products were observed, and denaturing polyacrylamide gel analysis (Figure 8B) indicated that these involved cleavages at T_{10}/A_{55}, T_{13}/A_{52}, and T_{16}/A_{49}. The structures of the three products are shown in Figure 5. Analysis of the partially cleaved products 2a and 3a (panel 8A) indicated primary sites of cleavage at positions T_{10}, T_{13}, T_{16}, and A_{55} (panel 8B). Thus, double-strand cleavage at T_{13}/A_{52} and T_{16}/A_{49} involved obligatory primary and secondary cleavage sites, but that at T_{10}/A_{55} did not.

The remaining seven hairpin DNAs in the library were also analyzed to establish their patterns of double-strand cleavage by Fe(II)·BLM A_5. Hairpin DNA **1** was cleaved at a total of 14 positions, including six sites within the original randomized region used to create the initial library (SI Figure S1),[12] but the analysis of double-strand cleavage revealed only a single double-strand cleavage product by native gel electrophoresis (SI Figure S2A). Sequencing gel analysis (SI Figure S2B) revealed that bands 2b and 3b in SI Figure S2A actually contained two sets of products putatively resulting from cleavage at A_9/T_{56} and T_{10}/A_{55}. Neither appeared to have resulted from coupled double-strand cleavage, nor was either within the original randomized region of the DNA hairpin library.

The analysis of double-strand cleavage of hairpin DNA **3** is shown in SI Figure S3. Two bands were apparent by the native polyacrylamide gel analysis (SI Figure S3A). Further analysis of each band by denaturing polyacrylamide gel electrophoresis (SI Figure S3B) indicated that one band had arisen by coupled double-strand cleavage at T_{15} and C_{49} and that T_{15} represented the primary site of cleavage. The other double-strand cleavage again involved T_{10} and A_{55}; this cleavage reaction did not appear to have an initial preferred site of cleavage.

Following cleavage with Fe(II)·BLM A_5, hairpin DNA **4** gave two double-strand cleavage bands when analyzed by non-denaturing polyacrylamide gel electrophoresis (SI Figure S4A). Each of the bands was isolated and then analyzed to determine the sites of cleavage by the use of a 16% denaturing polyacrylamide gel (SI Figure S4B). The upper bands from the native gel (2b and 3b) contained a linear duplex DNA resulting from cleavage at A_{12} and T_{53}. Analysis of bands 2a and 3a suggested that neither of these cleavage sites was a preferred site for the initial cleavage event. Analysis of the DNAs in bands 2c and 3c revealed that each contained DNAs with two cleavage sites. As was found for DNA **1**, the cleavage sites were putatively A_9/T_{56} and T_{10}/A_{55}. As for DNA **1**, neither of these cleavages appeared to involve a coupled event.

Hairpin DNA **5** had been found previously to undergo cleavage at 27 sites (SI Figure S1)[15] such that there were numerous possibilities for double-strand cleavage sites. Despite the abundance of single cleavage sites, treatment of DNA **5** with Fe(II)·BLM A_5 produced only three new bands (b, c, and d) following analysis on a native polyacrylamide gel (Figure S5A). Further analysis of each of these bands on a denaturing polyacrylamide gel (SI Figure S5B) indicated that each of these bands contained a single duplex resulting from double-strand cleavage. One of these resulted from cleavage at C_{12} and T_{54}, with the latter being the primary cleavage site. It may be noted that the

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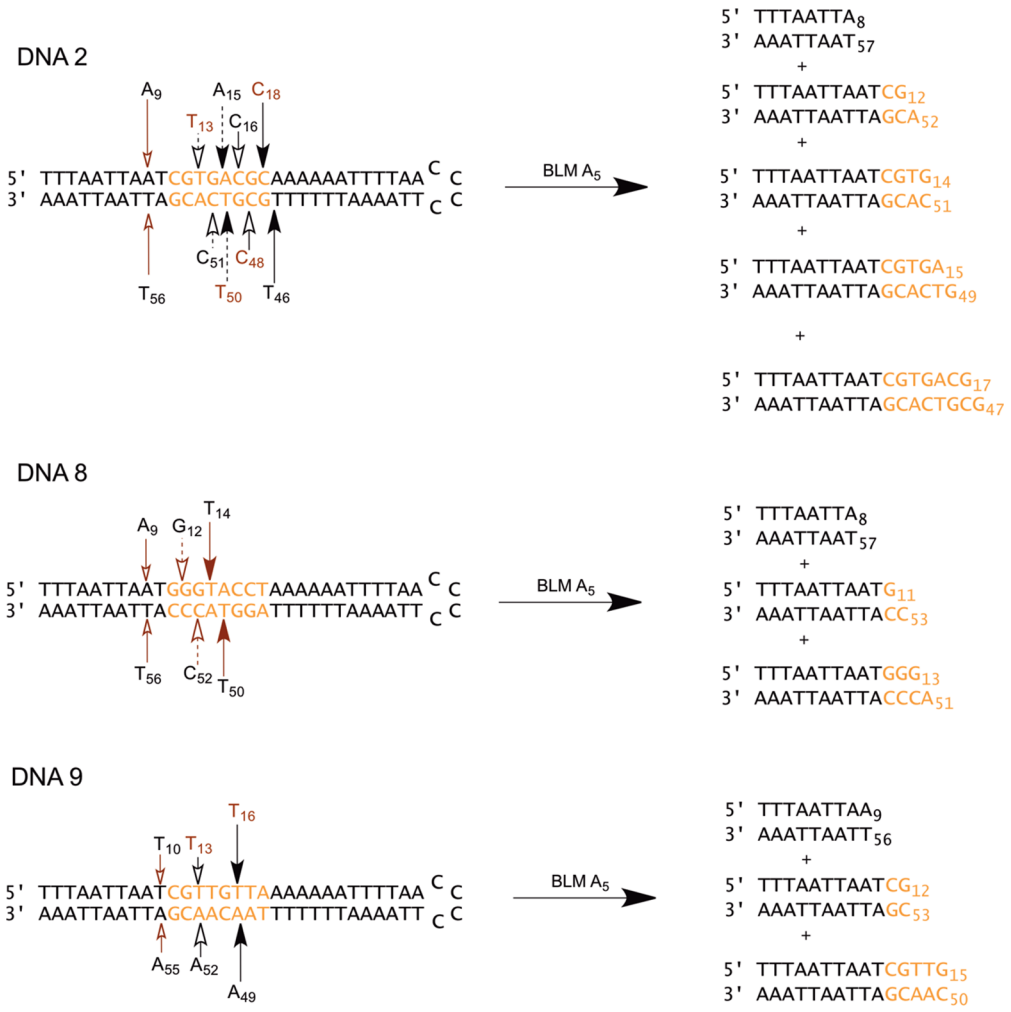

Figure 5. Summary of Fe·bleomycin-induced double-strand cleavage sites on DNAs **2**, **8**, and **9**. Orange bases indicate randomized region of the hairpin DNA library. Arrows of the same shape and color indicate paired cleavages. Black arrows correspond to coupled double-strand cleavage events, whereas red arrows indicate noncoupled double-strand cleavage events, resulting from two independent single-strand cleavages on opposite strands. Nucleotides colored in red indicate primary sites of coupled double-strand DNA cleavage.

orientation of these two nucleotides differed from those of all other coupled cleavages, an observation that was verified in replicate experiments. The other two products resulted from cleavage at T_{10}/T_{56} and A_{15}/T_{49}. It appeared that neither of these involved the obligatory use of one of the two cleavage sites for the primary cleavage event.

The study of hairpin DNA **6** is summarized in SI Figure S6. Two new double-strand cleavage products were apparent in SI Figure S6A, and these were analyzed further following isolation of each of the bands from the native gel. One of the double-strand cleavage products was found to have resulted from coupled cleavage at T_{12} and T_{52}, with the latter being the primary site of cleavage. The second double-strand cleavage product was formed by cleavage at A_9 and T_{56}, apparently without a preferred site for a primary cleavage reaction.

Treatment of hairpin DNA **7** with Fe(II)·BLM A_5 afforded five double-strand cleavage products, as revealed by subsequent analysis on a native polyacrylamide gel (SI Figure S7A). As for the other DNAs studied, each of the products was isolated from the native gel and analyzed on a denaturing polyacrylamide gel (SI Figure S7B). Three of the duplex products resulted from coupled double-strand cleavage events. These included a product formed by cleavage at T_{11} and T_{53}, for which the cleavage at T_{53} represented the primary site of cleavage. A second product involved cleavage at C_{13} and C_{51}, with the primary event involving cleavage at C_{51}. The third product involved initial cleavage at C_{17}, followed by cleavage at T_{47}. There were also two double-strand cleavage products for which no primary site of cleavage was apparent. These involved cleavage at T_{10}/A_{55} and C_{15}/C_{49}.

Regarding hairpin DNA **10**, treatment with Fe(II)·BLM A_5 afforded three double-strand cleavage products, as judged by the gel shown in SI Figure S8A. These were analyzed individually on a denaturing polyacrylamide gel (SI Figure S8B), which indicated the presence of two duplex DNAs formed by coupled double-strand cleavage. The cleavage sites that resulted in the formation of these two products were at C_{11}/C_{53} (primary cleavage site) and at C_{13} (primary cleavage site)/G_{52}. The third product, not formed by a coupled cleavage process, involved hairpin DNA cleavage at T_{10} and A_{55}.

The sites of coupled and noncoupled cleavage for the 10 hairpin DNAs considered in the present study are summarized in Figure 9. The sequence selectivity of cleavage of each appears in Tables 1 and 2 and is considered in some detail below.

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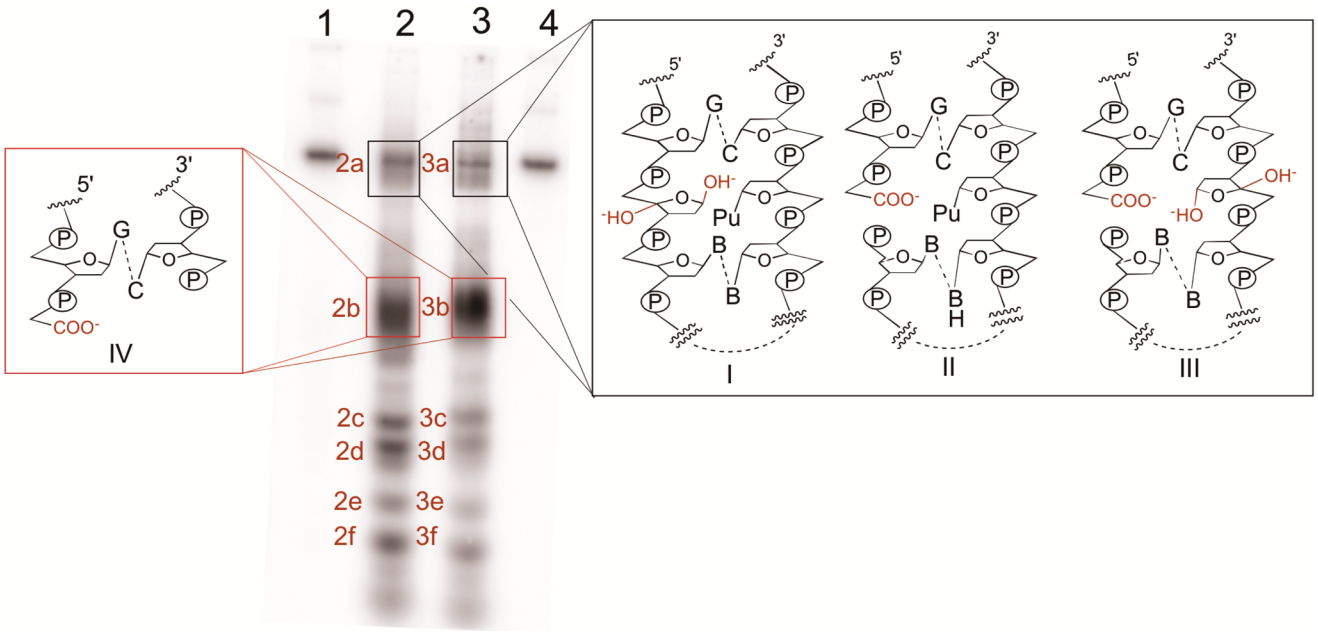

Figure 6. Double-strand cleavage of [3′-^{32}P]-end-labeled (lane 2) and [5′-^{32}P]-end-labeled (lane 3) 64-nucleotide hairpin DNA **2** by Fe·BLM A_5. Lane 1, [3′-^{32}P]-end-labeled DNA alone; lane 2, 5 μM Fe(II)·BLM A_5; lane 3, 5 μM Fe(II)·BLM A_5; lane 4, [5′-^{32}P]-end-labeled DNA alone.

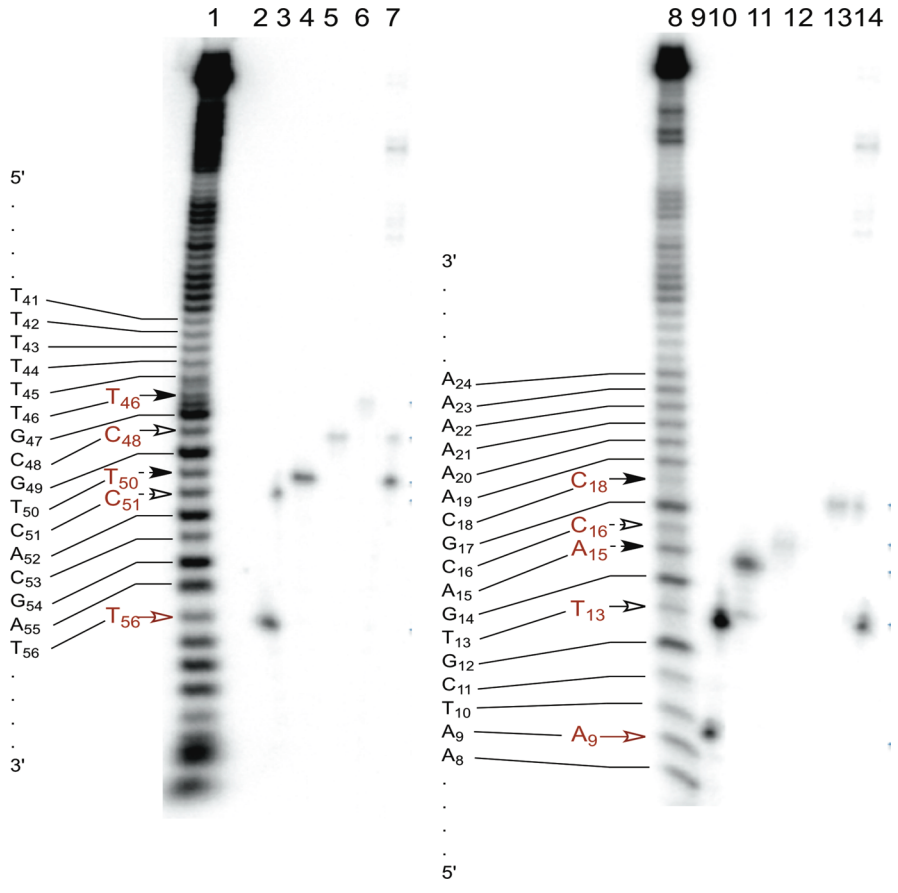

Figure 7. Sequencing gel analysis of Fe·bleomycin-induced double-strand cleavage sites of [3′-^{32}P]-end-labeled (lanes 1−7) and [5′-^{32}P]-end-labeled (lanes 8−14) hairpin DNA **2**. Each lane (except lanes 1 and 8) corresponds to a numbered cleavage band shown in Figure 6. Lane 1, Maxam−Gilbert G+A sequencing lane of [3′-^{32}P]-end-labeled DNA **2**; lane 2, band 2f; lane 3, band 2e; lane 4, band 2d; lane 5, band 2c; lane 6, band 2b; lane 7, band 2a; lane 8, Maxam−Gilbert G+A sequencing lane of [5′-^{32}P]-end-labeled DNA **2**; lane 9, band 3f; lane 10, band 3e; lane 11, band 3d; lane 12, band 3c; lane 13, band 3b; lane 14, band 3a.

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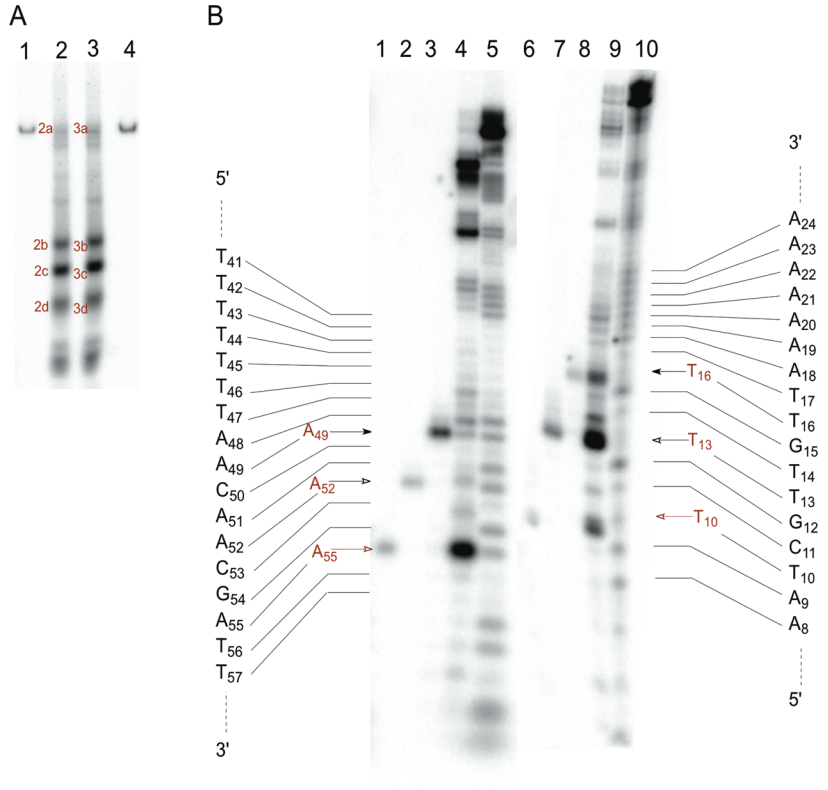

Figure 8. Analysis of bleomycin-induced double-strand cleavage sites on hairpin DNA **9**. (A) Double-strand cleavage of [3′-^{32}P]-end-labeled (lane 2) and [5′-^{32}P]-end-labeled (lane 3) 64-nucleotide hairpin DNA **9** by Fe·bleomycin A_5. Lane 1, [3′-^{32}P]-end-labeled DNA alone; lane 2, 5 μM Fe(II)·BLM A_5; lane 3, 5 μM Fe(II)·BLM A_5; lane 4, [5′-^{32}P]-end-labeled DNA alone. (B) Sequencing gel analysis of Fe·bleomycin-induced double-strand cleavage sites of [3′-^{32}P]-end-labeled (lanes 1−5) and [5′-^{32}P]-end-labeled (lanes 6−10) hairpin DNA **9**. Each lane (except lanes 5 and 10) corresponds to a numbered cleavage band, shown in (A). Lane 1, band 2d; lane 2, band 2c; lane 3, band 2b; lane 4, band 2a; lane 5, Maxam−Gilbert G+A sequencing lane of [3′-^{32}P]-end-labeled DNA **9**; lane 6, band 3d; lane 7, band 3c; lane 8, band 3b; lane 9, band 3a; lane 10, Maxam−Gilbert G+A sequencing lane of [5′-^{32}P]-end-labeled DNA **9**.

DISCUSSION

Despite decades of clinical use as an antitumor agent, the mechanism by which bleomycin exerts its therapeutic effects has never been established. Further, the exceptionally low clinical dose suggests the need for one or more highly specific mechanisms to achieve selective tumor cell killing. Recent studies have established the molecular basis for tumor cell targeting by bleomycin,[29] but not the mechanism of tumor cell killing. Presently, we analyze the interaction of Fe(II)·BLM with hairpin DNAs selected for tight binding to the drug and confirm our earlier finding that hairpin DNAs having a single strong binding site can be cleaved in many places.[13] Critically, we find that the lesions include numerous closely spaced double-strand cleavages, suggesting a mechanism for tumor cell killing.

Double-strand cleavage of DNA by bleomycin was first studied systematically by the Povirk laboratory.[28,30−33] In a study involving three linear DNA duplexes in which ∼250 base pairs were analyzed, they found a total of 26 double-strand DNA cleavage sites. Following treatment with Fe(II)·BLM A_2, the double-strand cleavage sites were identified by initial separation of the fragments on a nondenaturing polyacrylamide gel. An identical pattern was observed for 5′- and 3′-^{32}P-end-labeled DNAs, as would be expected for double-strand breaks. Subsequent analysis of each of the bands was carried out using a sequencing gel. With a single exception, each double-strand break involved cleavage at a G-Py sequence on one strand, but a much greater diversity of cleavage sequences was observed on the other strand. Eleven of the double-strand breaks resulted in blunt-end products, and 12 of the breaks afforded products having a one-nucleotide 5′-extension; no 3′-extensions were noted.[28]

Given that the DNA cleavage experiments carried out by Povirk et al.[28] were carried out under conditions of single-hit kinetics, the authors concluded that the double-strand breaks must have resulted from the action of a single BLM molecule. The finding of breaks at a G-Py sequence to produce virtually all of the observed double-strand cleavage products suggested that this must have been the primary site of cleavage. These workers also observed that in addition to double-strand breaks, alkali-labile lesions were also sometimes formed at the putative secondary cleavage sites. Enhanced cleavage opposite an initial BLM-induced nick was also supported by the work of Keller and Oppenheimer.[34] In subsequent studies, Absalon et al.[35,36] utilized hairpin DNA oligonucleotides to obtain much more detailed information about the nature of double-strand DNA cleavage. By the use of hairpin DNAs bearing an internal ^{32}P-labeled phosphate within the hairpin DNA, they were able to determine the ratio of single- to double-strand cleavage at individual sites. They also employed hairpin DNA substrates in which the C-4′ H atoms susceptible to abstraction by Fe·BLM had been replaced by deuterium. In agreement with the reports from the Povirk laboratory, they concluded that a single molecule of BLM effects double-strand DNA cleavage and requires reactivation after the first cleavage event for that purpose.

We have previously described a hairpin DNA that undergoes double-strand cleavage with very high efficiency at one site.[37]

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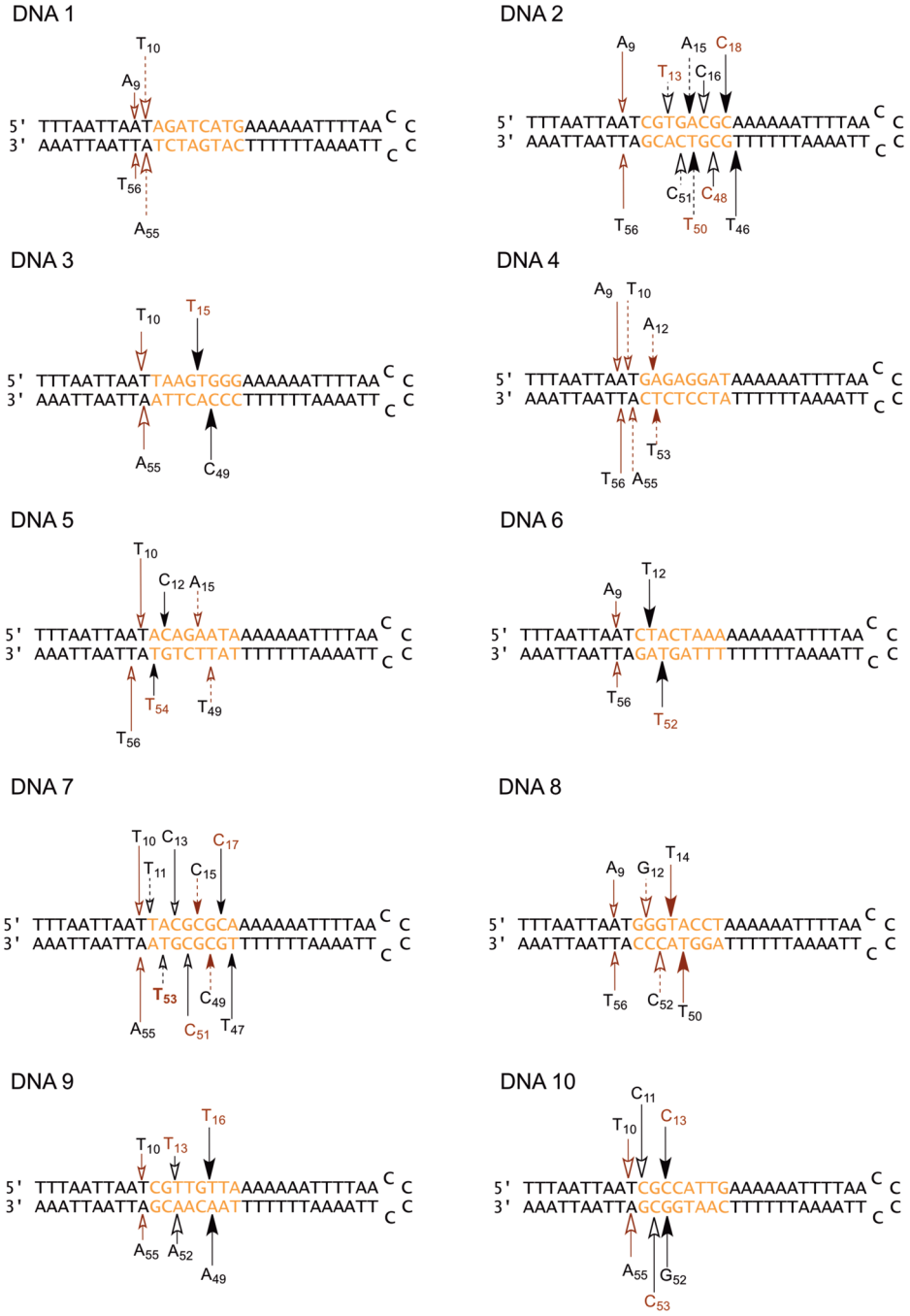

Figure 9. Summary of Fe·bleomycin-induced double-strand cleavage sites on hairpin DNAs **1**−**10**. Orange bases indicate randomized region of the original hairpin DNA library. Arrows of the same shape and color indicate paired cleavages. Black arrows correspond to coupled double-strand cleavage events, whereas red arrows indicate noncoupled double-strand cleavage events, which result from two independent single-strand cleavages on opposite strands. Nucleotides colored in red indicate primary sites of double-strand cleavage.

The strategy used for the assessing double-strand cleavage involved the introduction of a ^{32}P radiolabel alternatively at the 5′ or 3′-end of the hairpin DNA. For coupled events that occur in an obligatory order, the ratio of cleavage observed at the two sites will differ dramatically for the hairpin DNAs radiolabeled at the two ends. In fact, for the hairpin studied, the extent of cleavage at the two nucleotides constituting the preferred site cleavage was a strong function of the end of the DNA that had been radiolabeled, and this enabled detailed quantification of the extent of frank strand scission and alkali-labile products formed at the site of interest.

In the present case, the alternatively 5′- and 3′-end-labeled DNA fragments separated initially on the native gels also gave bands of different intensities when run on denaturing gels, providing confirmation that some of the double-strand cleavages observed were formed during coupled events. However, the large number of double-strand cleavage sites on some of the hairpin DNAs complicated an in-depth quantitative analysis of each site and prompted us to adopt a modified version of the strategy employed by Povirk et al.[28] Specifically, in addition to analyzing the individual bands containing double-strand cleavage products, we also analyzed the bands at the top of the gel that comigrated

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Table 2. Noncoupled Double-Strand BLM Cleavage Sites in Hairpin DNAs 1−10[a]

Hairpin DNA	Sequences cleaved
DNA 1 5′ AATAGATCATGAA 3′ TTATCTAGTACTT	AATA TTAT ATAG TATC
DNA 2 5′ AATCGTGACGCAA 3′ TTAGCACTGCGTT	AATC TTAG
DNA 3 5′ AATTAAGTGGGAA 3′ TTAATTCACCCTT	ATTA TAAT
DNA 4 5′ AATGAGAGGATAA 3′ TTACTCTCCTATT	AATG TTAC ATGA TACT GAGA CTCT
DNA 5 5′ AATACAGAATAAA 3′ TTATGTCTTATTT	ATAC TATG GAAT CTTA
DNA 6 5′ AATCTACTAAAAA 3′ TTAGATGATTTTT	AATC TTAG
DNA 7 5′ AATTACGCGCAAA 3′ TTAATGCGCGTTT	ATTA TAAT GCGC CGCG
DNA 8 5′ AATGGGTACCTAA 3′ TTACCCATGGATT	AATG TTAC GGGT CCCA GTAC CATG
DNA 9 5′ AATCGTTGTTAAA 3′ TTAGCAACAATTT	ATCG TAGC
DNA 10 5′ AATCGCCATTGAA 3′ TTAGCGCTAACTT	ATCG TAGC

[a]All sequences aligned from 5′-end of hairpin DNAs.

with intact hairpin DNA. As shown in Figure 3, these bands were anticipated to contain products with single-strand lesions, one of which (III) was a product containing frank strand scission on one strand and an alkali-labile lesion on the second strand in a position opposite the strand break. Product III was suggested by Povirk et al.[28] to result from partitioning of cleavage of the secondary DNA site by reactivated Fe·BLM into an alkali-labile product rather than a second strand break. Thus, product III should reliably identify the DNA strand containing the primary site of double-strand cleavage. As shown in Figure 2A, treatment of the putatively nicked full-length hairpin DNAs (2a and 4a) isolated from the native gel with *n*-butylamine did result in additional double-strand cleavage products (lanes 3 and 5), verifying the presence of products of type III in the residual full length hairpin DNAs following treatment with Fe·BLM.

Coupled double-strand DNA cleavage events were identified by analyzing the residual full length DNA in each lane. For products of type III (Figure 3), the DNA strand containing the primary cleavage site leading to coupled double-strand cleavage gave a band on the subsequent denaturing gel at the primary site of cleavage. The strand containing the alkali-labile lesion gave no corresponding band. This is illustrated in Figure 8B. Lanes 1, 2, and 3 illustrate the 3′-^{32}P-end-labeled DNAs recovered from bands 2d, 2c, and 2b, respectively, of the native gel shown in Figure 8A. The bands were formed by Fe·BLM-mediated cleavage at A_{55}, A_{52}, and A_{49}, respectively. Lane 4 contains the DNA isolated from band 2a. It contains a strong band corresponding to cleavage at A_{55}, but only very weak cleavage bands at A_{52} and A_{49}. Lanes 6, 7, and 8 illustrate the 5′-^{32}P-end-labeled DNAs recovered from bands 3d, 3c, and 3b, respectively. The bands were formed by Fe·BLM-mediated cleavage at T_{10}, T_{13}, and T_{16}, respectively. Lane 9 contains the DNA isolated from band 3a, which contains strong bands corresponding to cleavage at T_{10}, T_{13}, and T_{16}. The absence of strong bands at A_{52} and A_{49} in the DNA derived from the BLM-treated 3′-^{32}P-end-labeled full length hairpin DNA suggested that these sites contained alkali-labile lesions and that the double-strand cleavage at T_{13}/A_{52} and T_{16}/A_{49} is coupled, with T_{13} and T_{16} representing the primary sites of cleavage. The appearance of bands corresponding to A_{55} in lane 4 and T_{10} in lane 9 suggests one of two possibilities: namely, that (i) either of these sites can act as the primary site for coupled double-strand cleavage, or (ii) the double-strand cleavage at T_{10}/A_{55} results from two independent cleavage events. Because the double-strand cleavages were not carried out under conditions rigorously intended to ensure single-hit kinetics, we cannot be certain whether the cleavage at T_{10}/A_{55} involved a single BLM molecule. However, on the basis of observations made below about the nature of the cleavage of these strongly bound hairpin DNAs, we believe that the cleavage at T_{10}/A_{55} and other similar cleavages likely represent two independent events.

In their study of double-strand DNA cleavage, Povirk et al.[28] reported that for all but one of 26 double-strand cleavage sites, one cleavage involved a G-Py cleavage, but the other strand rarely involved G-Py cleavage and usually involved a sequence for which single-strand cleavage was unusual. Although G-Py was not confirmed to be the primary cleavage site, in the present study, all 14 confirmed coupled double-strand cleavages involved initial cleavage of a G-Py sequence. In the earlier study, seven of the 26 double-strand cleavages involved the first Py residue of G-Py-Py-Pu sequences, and in all cases, the cleavage on the opposite strand afforded blunt-end products. Six examples of double-strand cleavage at the first Py of G-Py-Py-Py sequences were also noted, and cleavage on the opposite strand afforded either blunt-end products or products having a single-base 5′ extension on the opposite strand. As shown in Table 1, for the 14 confirmed coupled double-strand cleavages in the present study, there were four involving G-Py-Py-Pu sequences, but none involving G-Py-Py-Py sequences.

The earlier study also found that six of the double-strand cleavages occurred at G-Py-Pu-Py sequences and three at G-Py−Pu-Pu sequences. In all cases, cleavage on the opposite strand afforded duplexes with one-base 5′ extensions. In comparison, in the current study, three coupled double-strand cleavage events involved G-Py-Pu-Py sequences, with two producing products

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having one-base 5′ extensions, and the other, a 3′ one-base extension (Table 1). Interestingly, there were seven examples of double-strand cleavage involving G-Py-Pu-Pu sequences, all of which gave products with one-base 5′ extensions (Table 1). Thus, the present findings closely parallel the report by Povirk et al.[28] regarding the primary cleavage site for coupled double-strand cleavage of DNA by Fe·BLM and the production of blunt-end products or those having one-base 5′ extensions. The overall frequency of coupled double-strand cleavages in the two studies was also reasonably similar, although somewhat greater for the strongly bound hairpin DNAs in the current study. The earlier study found 26 double-strand cleavages in ∼250 base pairs, and the present findings involved 14 coupled double-strand cleavages largely within the variable (eight nucleotide) regions of 10 DNAs, a total of ∼80 base pairs. The reasons for the variations in recognition of specific four-base-pair sequences is less clear, but it could be due in part to the relatively small number of sequences sampled.

In comparison with the coupled double-strand cleavages summarized in Table 1, the 17 noncoupled double-strand cleavages outlined in Table 2 are unprecedented. None were observed in the earlier study by Povirk et al.;[28] neither has a phenomenon of this type been reported elsewhere. No clear sequence preference for cleavage is apparent, other than the frequency of appearance of AT-rich sequences. Many of these cleavages occurred at the junctions between the conserved and variable regions of the hairpin DNAs, which are AT-rich and so may be regarded as a consequence of experimental design. However, apparently noncoupled double-strand cleavages included a GAGA sequence in DNA **4**, a GAAT sequence in DNA **5**, a GCGC sequence in DNA **7**, and both GTAC and GGGT sequences in DNA **8**.[38]

In a recent report, we have used surface plasmon resonance to study the dynamics of Fe(III)·BLM binding to three of the hairpin DNAs employed in the present study (**2**, **4**, and **5**).[13] Each of these DNAs was found to have a single strong binding site for Fe(III)·BLM, but one or more significantly weaker binding sites. In contrast, each was found to undergo cleavage at a number of sites, affording multiple single- and double-strand cleavage products (SI Figure S1 and Figure 9). For hairpin DNA **2**, a competition experiment was also carried out assessing the ability of a 16-nucleotide hairpin DNA known to be a good substrate for cleavage by Fe·BLM to compete with hairpin DNA **2** for a limited amount of Fe·BLM. Although the SPR measurements demonstrated that Fe·BLM was capable of dissociating from DNA **2**, in the presence of a single equivalent of this DNA, no cleavage of the 16-nt hairpin DNA substrate could be observed.[13]

The model that emerges from these measurements is one in which Fe·BLM is closely associated with the strongly bound hairpin DNAs, even when not bound to them as judged by surface plasmon resonance and even when other substrate DNAs are present. This persistent association should permit ample opportunity for Fe·BLM to effect strand scission of the hairpin DNA repeatedly, potentially leading to the multiple single- and double-strand cleavage products actually observed. In this context, it may be noted that hairpin DNAs **2** and **7**, each of which underwent five closed spaced double-strand DNA cleavage reactions, had previously been shown to bind to Fe·BLM the most tightly of the 10 hairpin DNAs in the library used for the present study, whereas hairpin DNAs **1**, **3**, and **6**, each of which underwent only two double-strand DNA cleavages, were among those bound least strongly to Fe·BLM.[12] Chromosomal DNA presumably contains regions of DNA capable of binding Fe·BLM significantly more tightly than any of the DNAs employed in the present study. If the pattern of DNA cleavage observed here proves to be more general, one might expect such regions to undergo extensive double-strand cleavage, potentially leading to significant gaps in the chromatin structure. Such gaps would presumably be difficult to repair and might well compromise cell viability.

The hairpin DNAs employed in the present study were selected for their ability to bind tightly to BLM, but are certainly not optimal in that regard. In addition, it is not clear which specific structural elements in the hairpin DNAs are responsible for the tight binding of BLM. Experiments intended to address these issues and thereby enable further understanding of the possible role of tight DNA binding in the expression of antitumor activity by bleomycin are underway.

EXPERIMENTAL METHODS

Materials. T4 polynucleotide kinase was purchased from New England Biolabs. Recombinant terminal deoxynucleotidyl transferase was obtained from Roche. [γ-^{32}P]ATP and [α-^{32}P]ddATP were purchased from Perkin-Elmer. $Fe(NH_4)_2(SO_4)_2{\cdot}6H_2O$ and Chelex 100 were from Sigma Aldrich. The hairpin DNAs were obtained from Integrated DNA Technologies, Inc.

[5′-^{32}P]-End and [3′-^{32}P]-End-Labeling and Purification of Hairpin DNAs. The hairpin DNAs were end-labeled using [γ-^{32}P]ATP + T4 polynucleotide kinase and [α-^{32}P]ddATP + terminal deoxytransferase at the 5′ and 3′ ends, respectively. Ten picomoles of 64-nucleotide hairpin DNAs were [5′-^{32}P]-end-labeled by incubation with 20 units of T4 polynucleotide kinase and 0.06 mCi [γ-^{32}P]ATP (specific activity 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 DNA purification by 16% polyacrylamide gel electrophoresis at 1800 V for 2.5 h. The 3′-end-labeling was done by incubating 10 pmol of hairpin DNA with 20 units of terminal deoxynucleotidyl transferase and 0.06 mCi [α-^{32}P]ATP (specific activity 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′-^{32}P]-End and [3′-^{32}P]-End-Labeled Hairpin DNAs by Bleomycin A_5. Bleomycin cleavage of [5′-^{32}P]- and [3′-^{32}P]-end-labeled hairpin DNAs was performed by incubating the hairpin DNA (∼30 000 cpm) with 5 μM Fe^{2+} and 5 μM bleomycin A_5 at 25 °C for 30 min in a solution containing 10 μL of 2 mM $MgCl_2$ and 10 mM Na cacodylate, pH 7.0. Two microliters of native gel loading buffer containing 0.25% bromophenol blue, 0.25% xylene cyanol, and 40% D-sucrose were 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 gels using a phosphorimager.

***n*-Butylamine Treatment of 64-Nucleotide Hairpin DNA 8.** Bleomycin cleavage reactions of [5′-^{32}P]-end and [3′-^{32}P]-end-labeled DNA **8** were further treated with 2 mM *n*-butylamine and incubated at 25 °C for 10 min. The supernatants were removed under diminished pressure, and the DNA pellets were washed with 10 μL of deionized water. The final solutions were mixed with 2 μL of native gel loading buffer containing 0.25% bromophenol blue, 0.25% xylene cyanol, and 40% D-sucrose and separated on 20% native polyacrylamide gels (200 V, 16 h, 4 °C).

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Maxam–Gilbert Sequencing Reaction.[39] Ten microliters of [5′-^{32}P]- and [3′-^{32}P]-end-labeled DNAs (~50 000 cpm) were treated with 25 μL of formic acid and incubated at 25 °C for 4–5 min. The reactions were stopped by treatment with 200 μL of 0.3 M NaOAc, pH 7.0, 0.1 mM EDTA, and 25 μg/mL tRNA. The resulting solutions were mixed with 700 μL of ethanol, and the DNAs were precipitated. The DNA pellets were washed twice with 70% ethanol, and the pellets were resuspended in 75 μL of 10% piperidine. The reaction mixtures were incubated at 90 °C for 30 min, and 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′-^{32}P]-end- and [3′-^{32}P]-end-labeled DNAs on denaturing polyacrylamide gels.

Denaturing Gel Electrophoresis of DNA Cleavage Products. The [5′-^{32}P]-end- and [3′-^{32}P]-end-labeled double-strand DNA cleavage bands visualized by native gel electrophoresis were excised from the gels and purified by ethanol precipitation and then mixed with 5 μL of denaturing gel loading buffer containing 80% formamide, 2 mM EDTA, 1% bromophenol blue, and 1% xylene cyanol, then heated at 90 °C for 10 min. Five microliters of the final solutions were chilled on ice and separated on a 16% denaturing polyacrylamide gel containing 16% urea along with 2 μL of [5′-^{32}P]- and [3′-^{32}P]-end-labeled Maxam–Gilbert sequencing lanes to determine the sequences of the cleavage sites. The gels were visualized using a phosphorimager.

■ ASSOCIATED CONTENT

Supporting Information

High-resolution polyacrylamide electrophoresis gels for several hairpin DNAs and a summary of all previously reported cleavage sites for the 10 hairpin DNAs. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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