Kinetics of DNA Cleavage by Fe(II)·Bleomycins

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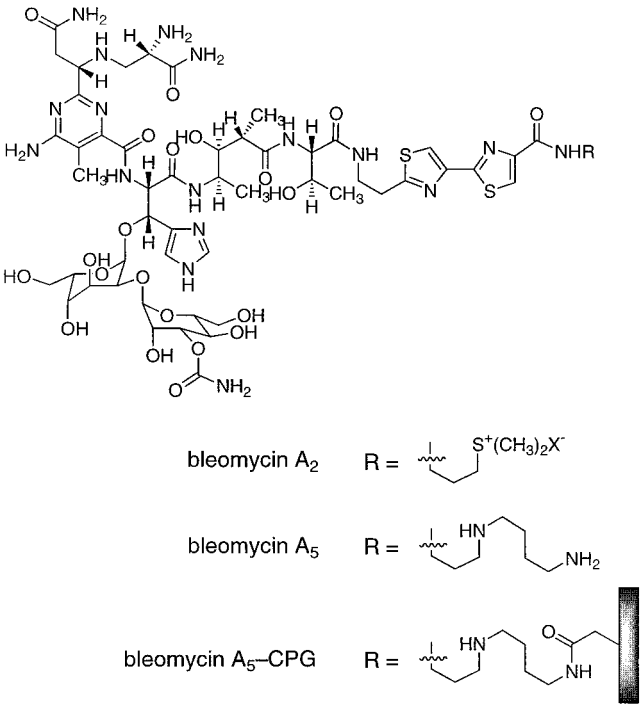
The bleomycins (BLMs) are glycopeptide-derived antitumor antibiotics employed clinically for the treatment of certain forms of cancer.[1] The antitumor properties of BLM are believed to result from the ability of the drug to effect the oxidative degradation of DNA,[2] and possibly also of RNA.[3] In the presence of metal ions such as Fe^{2+}, bleomycin forms a binary complex (Fe(II)·BLM) that can activate molecular oxygen, forming a ternary complex that is capable of polynucleotide degradation.[2,4] The chemistry of nucleic acid degradation by these species has been the subject of numerous studies;[2,5] less attention has been given to the actual rates of product formation, reflecting the reality that degradation is rapid, while the analysis of product formation is time-consuming. The time course of Fe(II)·BLM A_2-mediated DNA degradation was studied previously using reversed-phase HPLC,[4a] but the measurement of reaction kinetics was not possible. To better understand the kinetic properties of BLM-mediated DNA cleavage, a new strategy has been developed.

We have applied the molecular beacon concept for monitoring the kinetics of Fe(II)·BLM-mediated DNA cleavage. A molecular beacon is an oligonucleotide hairpin with attached fluorescence donors and quenchers at its 5′- and 3′-termini.[6] Changes in the spatial proximity of the donor and acceptor, for example through beacon cleavage[7] or conformational changes in the beacon, can be determined readily via changes in the efficiency of fluorescence resonance energy transfer (FRET).[8] Presently, we report the (i) real-time measurement of BLM A_2-mediated beacon DNA degradation by fluorescence spectroscopy, (ii) analysis of the formed products, permitting the actual kinetics of DNA cleavage to be calculated, and (iii) visualization of DNA cleavage by BLM A_5 conjugated to controlled pore glass beads.



As shown in Figure 1, the molecular beacon contained 16 nucleotides capable of forming a six base-pair stem. The stem

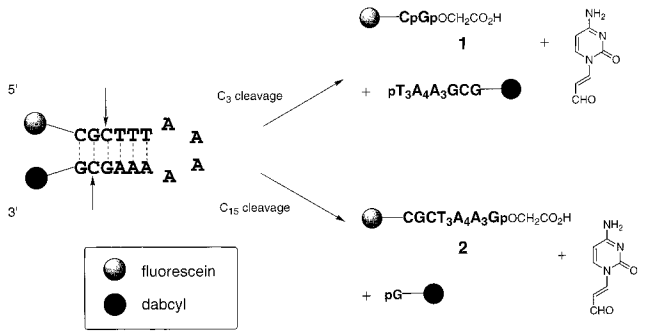


Figure 1. Structure of the molecular beacon used for bleomycin-mediated DNA cleavage, and possible degradation products.

sequence was designed to mimic the self-complementary dodecanucleotide d($CGCT_3A_3GCG$), which is a highly efficient substrate for Fe(II)·BLM.[5] By analogy with d($CGCT_3A_3GCG$) cleavage, degradation of the beacon was expected to take place almost exclusively at C_3 and C_{15} to afford fluorescein-linked phosphoroglycolate products (**1** and **2**). The fluorophore fluorescein was attached to the 5′-end of the beacon via a hexamethylene linker, while the quencher dabcyl was attached to the 3′-end via a functionalized cytidine (Figure 1, Supporting Information). Stem formation by the beacon keeps these two moieties in close proximity to each other, causing the fluorescence to be quenched by FRET.[8] The fluorescence emission of fluorescein should be restored following bleomycin-mediated cleavage of the stem sequence and concomitant release of the fluorophore-linked products. This should permit the progress of beacon DNA cleavage to be monitored continuously by simply recording the increase in fluorescence with time.

The increase in fluorescence emission resulting from Fe(II)·BLM A_2-mediated beacon DNA cleavage was monitored by fluorescence spectroscopy.[9] Figure 2 shows the time course of the fluorescence increase of a fixed amount of beacon DNA treated with different concentrations of Fe(II)·BLM A_2. As shown in Figure 2, fluorescence emission increased as a function of bleomycin concentration, resulting in ∼10-fold increase in

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(9) Each cleavage reaction was carried out at 25 °C in 300 μL of 10 mM sodium cacodylate, pH 7.0. The samples were excited with 494 nm light, and the fluorescence emission was monitored at 518 nm.

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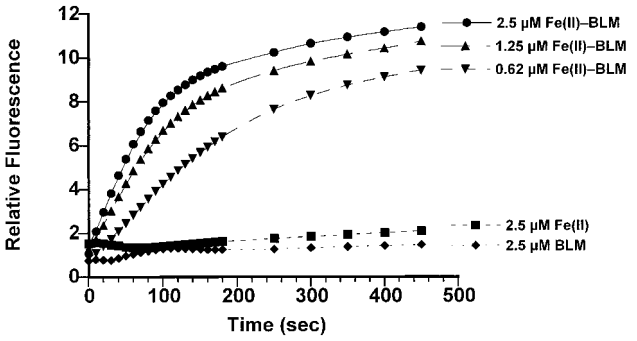

Figure 2. Time-dependent fluorescence emission intensity of beacon DNA treated with varying concentrations of Fe(II)·BLM A_2. The beacon DNA concentration was 0.72 μM.

fluorescence intensity. Beacon DNA cleavage was enhanced at higher concentrations of Fe(II)·BLM A_2, albeit not in proportion to the amount of BLM present. Bleomycin-mediated DNA cleavage was also monitored as a function of DNA concentration (not shown). With increasing DNA substrate concentration, the resulting fluorescence did not increase in proportion, suggesting non Michaelis−Menten cleavage kinetics.

The chemistry of Fe(II)·BLM A_2-mediated beacon DNA cleavage was studied to define the actual products. Reversed-phase HPLC analysis of the products resulting from the beacon DNA cleavage indicated the production of fluorescein oligonucleotide glycolate **2** and roughly equivalent amounts of cytosine propenal. While glycolate **2** was presumably formed from cleavage at C_{15}, the formation of dinucleotide **1**, resulting from cleavage at C_3, could not be detected.[10] This observation indicated that beacon DNA cleavage occurred exclusively at C_{15}, in agreement with the results for the dodecanucleotide substrate upon which the beacon DNA was designed.[5,11]

The increase in fluorescence is linearly related to the number of cleaved beacon DNA molecules, each of which affords an equivalent of oligonucleotide glycolate **2**.[5,12] Therefore, to determine the number of DNA cleavage events, the amount of **2** formed was determined.[14] The initial velocities of product formation were calculated to be 1.6, 1.2, and 0.66 nM DNA sec^{-1} from the initial phase of the curves for 2.5, 1.2, and 0.62 μM Fe(II)·BLM A_2, respectively.[15] By varying the concentration of Fe(II)·BLM A_2 such that the DNA substrate was saturated, the pseudo-first-order rate constant was determined to be $(2.39 \pm 0.33) \times 10^{-3}$ sec^{-1}.

The possibility of visualization of bleomycin-mediated beacon DNA cleavage was examined by fluorescence microscopy.

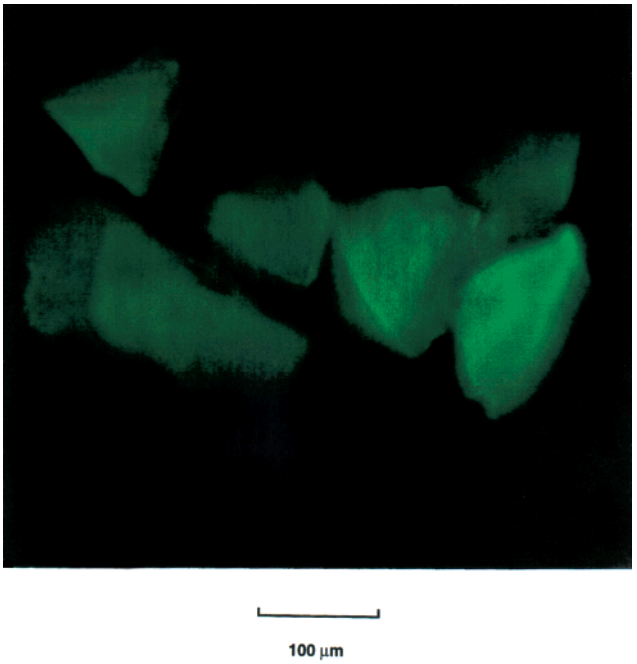

Figure 3. Fluorescence microscopy (30-min incubation) of the beacon DNA treated with Fe(II)·BLM A_5 −CPG.

Controlled pore glass bead (CPG)-conjugated BLM A_5 was employed for microscopic visualization.[16] Beacon cleavage for visualization was carried out with the beads embedded in an agarose gel medium to retard diffusion of the formed fluorescent products from the beads.[17] Figure 3 shows the fluorescence image of beacon DNA treated with activated, immobilized Fe(II)·BLM A_5. The green fluorescence emission from each of the conjugated beads could be observed within 2 min and persisted for more than 1 h. Thus, it was possible to show that the BLM attached to a specific bead was able to mediate (beacon) DNA cleavage.

Recently, we have described the solid-phase synthesis of a bleomycin congener on a solid support,[18] and we have also shown that the sequence selectivity and products of DNA cleavage by immobilized BLM A_5 were indistinguishable from those of free BLM A_5.[16] The finding that beacon cleavage can be monitored visually on individual beads argues that it should be possible to survey the DNA cleaving properties of individual BLM congeners prepared as members of libraries by split resin synthesis.

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Supporting Information Available: Chemical structures of fluorescein and dabcyl moieties of the molecular beacon DNA (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(10) Oligonucleotide **1** was synthesized in analogy to a published procedure.[5] Oligonucleotide **2** was prepared by T4 RNA ligase-mediated condensation of fluorescein-conjugated d($CGCT_3A_6$) and $ApGpOCH_2COOH$. Authentic **2** co-eluted on C_{18} reversed-phase HPLC with the sample of putative **2** isolated following treatment of the beacon DNA with Fe(II)·BLM A_2.

(11) Alteration of the cleavage ratio of d($CGCT_3A_3GCG$) at C_3 vs C_{11} with individual Fe(II)·BLM congeners has been noted previously (Sugiyama, H.; Kilkuskie, R. E.; Chang, L.-H.; Ma, L.-T.; Hecht, S. M.; van der Marel, G. A., van Boom, J. H. *J. Am. Chem. Soc.* **1986**, *108*, 3852). While the beacon DNA employed here was a good substrate for Fe(II)·BLM, it is interesting that complete consumption of the beacon DNA was not achieved at any concentration of Fe(II)·BLM employed (i.e., up to a 28-fold molar excess).

(12) BLM-mediated abstraction of C-4′H from a subset of DNA deoxyribose moieties has been shown to be rate-limiting for DNA degradation.[13] Accordingly, release of cleaved oligonucleotide was assumed not to be rate-limiting here.

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(14) The fluorescence signals measured upon formation of free **2** were quantified by comparison with a standard curve for authentic oligonucleotide glycolate **2**.[10]

(15) Quite recently the application of molecular beacons for monitoring DNA cleavage by nucleases and small DNA-cleaving molecules has been reported by the groups of Tan and Thorson.[7] The DNA cleavage by Fe(II)·bleomycin has also been investigated by the latter group, but the actual fluorescent products were not analyzed. Maximum fluorescence emission was simply assumed to correspond to complete consumption of the DNA substrate, an assumption that may not be justified in view of the present findings.[11]

(16) CPG-conjugated BLM A_5 was prepared according to the method reported previously (Abraham, A. T.; Zhou, X.; Hecht, S. M. *J. Am. Chem. Soc.* **1999**, *121*, 1982). The extent of derivatization was approximately 79 μg/mg of bead.

(17) To a mixture of 6.7 μM beacon DNA and 440 μM BLM A_5−CPG was added 2.4% agarose gel solution, and the combined mixture was maintained at 0 °C for 1 h. The mixture (total 80 μL) contained 2.5 μM beacon DNA, 170 μM BLM A_5−CPG in 1.5% agarose gel. To this agarose gel mixture was added 9 μL of 2 mM $Fe(NH_4)_2(SO_4)_2$ solution for activation of BLM A_5. The mixture was maintained at 0 °C for 2 min, and a gel piece containing conjugated CPG beads was excised from the gel solution. The gel piece was sandwiched between cover glasses and subjected to analysis by fluorescence microscopy. The sample was monitored through a FITC filter (following excitation at 480 ± 20 nm light; fluorescence emission was monitored 535 ± 25 nm).

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