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An Efficient Mammalian Transfer RNA Target for Bleomycin

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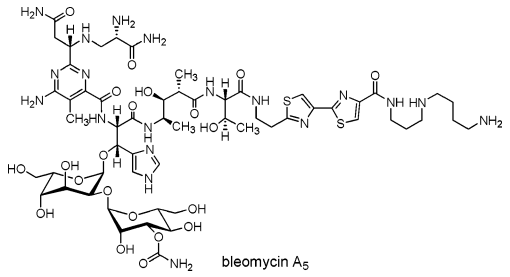
Bleomycin (BLM) is a clinically used antitumor agent, efficacious in the treatment of malignant lymphomas and squamous cell carcinomas.[1] While the antitumor action of bleomycin has been attributed to its oxidative degradation of DNA,[2] in recent years increasing attention has been focused on bleomycin-induced cleavage of RNA.[3] Fe·bleomycin has now been shown to cleave some members of all major classes of RNAs, including transfer RNAs, messenger RNAs, ribosomal RNAs, and the RNA strands of RNA−DNA heteroduplexes.[4] Recently, it has been shown that BLM can inhibit protein synthesis in an intact biological system (*Xenopus oocytes*) as well as in a cell free protein synthesizing system; inhibition results from the degradation of one or more transfer RNAs.[5] Since tRNAs highly susceptible to oxidative cleavage by BLM might bear relevance to the mechanism by which the drug exerts its antitumor effects, we have sought to define the most susceptible tRNA species. Presently, we report that two tRNA isoacceptors are cleaved with exceptional facility by Fe(II)·BLM. One of these, $tRNA_3^{Lys}$, undergoes ready cleavage under physiological conditions, predominantly at a single site.

Chicken liver is an abundant source of mammalian tRNAs, which have been shown to be closely homologous to human tRNAs in sequence.[6] Following initial separation by BD-cellulose chromatography and 10% polyacrylamide−4 M urea gel electrophoresis (PAGE),[7] 85 fractions enriched in single tRNAs were isolated and tested individually for their susceptibility to degradation by Fe(II)·BLM;[9] a representative gel is shown in the Supporting Information (Figure S1). Four fractions contained exceptionally good substrates for cleavage by Fe(II)·BLM, and these were purified to homogeneity by 15% denaturing PAGE.



Homochromatography sequence analysis[6] of two purified chicken liver tRNA isoacceptors indicated that they were $tRNA_3^{Lys}$ and $tRNA^{Phe}$. The ability of Fe(II)·BLM A_5 to cleave these species efficiently was verified using the purified tRNA isoacceptors (Figures 1 and S2 for $tRNA_3^{Lys}$ and $tRNA^{Phe}$, respectively), and analysis of the major site of cleavage (arrows) indicated that U_{66} was the site of cleavage in each tRNA (Figure 2). The susceptibility

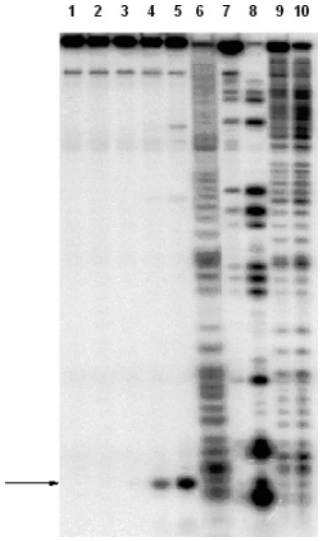


Figure 1. Chicken liver $tRNA_3^{Lys}$ cleavage by BLM A_5. 3′-^{32}P-Labeled chicken liver $tRNA_3^{Lys}$ was treated with Fe(II)·BLM A_5 and then analyzed by 15% PAGE. Lane 1, $tRNA_3^{Lys}$ alone; lane 2, 10 μM Fe^{2+}; lane 3, 10 μM BLM A_5; lane 4, 1 μM Fe(II)·BLM A_5; lane 5, 10 μM Fe(II)·BLM A_5; lane 6, aqueous NaOH; lane 7, RNase T1 (G); lane 8, RNase T1 (G); lane 9, RNase T2; lane 10, RNase T2.

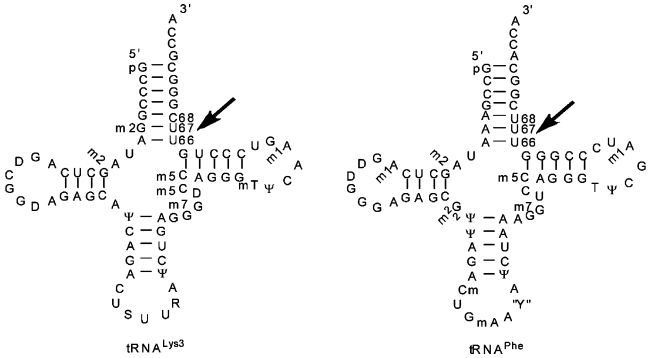


Figure 2. Primary structures of chicken liver $tRNA^{Phe}$ and $tRNA_3^{Lys}$. Arrows indicate the sites of cleavage by Fe(II)·BLM A_5.

of certain tRNA isoacceptors to cleavage by Fe(II)·BLM is diminished in the presence of Mg^{2+}; this has been shown explicitly for yeast $tRNA^{Phe}$.[10] Study of the Mg^{2+} sensitivity of the cleavage shown in Figures 1 and S2 revealed that added Mg^{2+} strongly diminished the cleavage of chicken liver $tRNA^{Phe}$ but was much less inhibitory toward $tRNA_3^{Lys}$ cleavage (Figure S3). In fact, as shown for a synthetic polynucleotide having the same nucleotide sequence as $tRNA_3^{Lys}$, albeit without modified nucleobases, cleavage by 10 μM Fe(II)·BLM was actually increased by the addition of Mg^{2+} up to 5.0 mM concentration (Figure 3), i.e., at physiological concentrations.[11] The altered cleavage by Fe(II)·BLM in the

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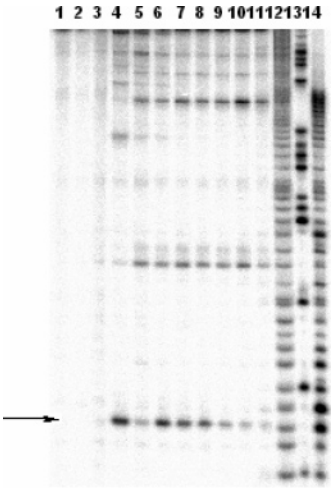

Figure 3. Cleavage of a synthetic oligonucleotide identical in sequence with $tRNA_3^{Lys}$ by Fe(II)·BLM. Lane 1, $tRNA_3^{Lys}$ alone; lane 2, 1.0 μM Fe^{2+}; lane 3, 1.0 μM BLM; lane 4, 1.0 μM Fe(II)·BLM; lane 5, 10 μM Fe(II)·BLM; lanes 6−11, 10 μM Fe(II)·BLM + 0.1, 0.5, 1.0, 1.5, 3.0, and 5.0 mM Mg^{2+}, respectively; lanes 12−14, sequencing lanes.

presence of added Mg^{2+} no doubt reflects the interaction of weakly bound Mg^{2+} with tRNA at millimolar concentrations of Mg^{2+}.

The cleavage of the tRNAs observed at U_{66} parallels earlier findings for the preferred position of tRNA cleavage sites.[3,4] Most of these sites are at the junction between single- and double-stranded regions of the RNA, where the minor grooves would be expected to be somewhat widened, potentially providing improved access for the binding of Fe(II)·BLM, which is large compared to a typical minor groove structure in DNA or RNA.[2,4]

It is instructive to consider the possible relevance of $tRNA_3^{Lys}$ as a therapeutic target for bleomycin. As described previously, BLM appears to exert its cytotoxicity toward cultured tumor cell lines by a mechanism similar to that employed by the cytotoxic antitumor ribonuclease onconase.[5,12,13] Onconase cannot cleave DNA and apparently exerts its antitumor effects at the level of protein synthesis inhibition by degrading one or more transfer RNAs.[5,12] Recently, it was reported that onconase degrades $tRNA_3^{Lys}$ and $tRNA^{Phe}$ in HIV-1-infected H9 lymphocytes.[14] Thus, two antitumor agents, believed to function by related mechanisms, both specifically degrade $tRNA_3^{Lys}$, and also $tRNA^{Phe}$, with great facility. While not definitive, these findings support the possibility that the antitumor activity of bleomycin may derive in part from the degradation of a critical RNA such as $tRNA_3^{Lys}$.

There is evidence that BLM can accumulate at high concentrations in both the nucleus and cytoplasm of mammalian cells,[5,15] such that competition between DNA and RNA for limiting amounts of Fe(II)·BLM is unlikely. Nonetheless, it may be noted that a tRNA cleaved efficiently by Fe(II)·BLM also bound the drug effectively and was cleaved in the presence of DNA.[3b,4,16]

Definition of the mechanism of antitumor action of bleomycin might well benefit from the identification of a BLM analogue more specific for the cleavage of an RNA such as $tRNA_3^{Lys}$. The cleavage of a synthetic polynucleotide having the same sequence as $tRNA_3^{Lys}$ by Fe(II)·BLM (Figure 3) may permit the selection of an RNA-selective BLM from a synthetic combinatorial library of bleomycins by obviating the need to isolate large amounts of the native tRNA.[17] Further, the construction of a synthetic RNA beacon based on the structure of $tRNA_3^{Lys}$ would clearly be helpful in this regard.[18]

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Supporting Information Available: Polyacrylamide gels illustrating the cleavage of tRNA fractions by Fe(II)·BLM. This material is available free of charge via the Internet at http://pubs.acs.org.

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(7) Fifteen fractions were collected from the benzoylated DEAE- (BD-) cellulose column, and these were 3′-^{32}P-end-labeled[8] and separated by electrophoresis on a 10% polyacrylamide−4 M urea gel at 4 °C.[6] tRNAs were isolated from 85 bands and then treated with 10 and 100 μM Fe(II)·BLM. Those fractions cleaved most efficiently, all of which involved the most hydrophobic fractions from the BD-cellulose column, were analyzed further with 1 and 5 μM Fe(II)·BLM. While these experiments were carried out in the absence of added Mg^{2+}, tRNAs are folded with tightly bound Mg^{2+}, and these Mg^{2+} were no doubt present.
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