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QUARTERLY

Modified RNAs as potential drug targets*°

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Bleomycin (BLM) is a natural antibiotic that is effective in treatment of selected cancers. Although the exact therapeutic mechanism of bleomycin is not known, its target is thought to be a nucleic acid. Besides cleaving DNA, *in vitro*, Fe-bleomycin cleaves the anticodon of yeast $tRNA^{Phe}$ specifically. Using CD and fluorescence spectroscopy we have found that apo-bleomycin binds to synthetic RNA analogs of the anticodon of yeast $tRNA^{Phe}$ with an affinity similar to that previously reported for DNA. In order to understand BLM's selectivity, the role magnesium ions play in RNA recognition should be explained. Many RNA substrates for Fe-BLM, including yeast $tRNA^{Phe}$, are not cleaved by the drug when the Mg^{2+} concentration exceeds 1 mM. Competition experiments with anticodon analogs provide insight into the role of magnesium ions in RNA recognition by BLM. These simple modified RNAs may be useful as model systems for investigating BLM/RNA recognition and development of highly selective drugs toward RNA targets.

The bleomycins (BLM, Fig. 1) are natural glycopeptide antibiotics [1] approved as antineoplastic agents for the treatment of carcinomas and lymphomas [2]. While administered in the apo form, their therapeutic effect is attributed to hydrolytic degradation of DNA [3] and possibly RNA [4] by the Fe-complexed form. Strong evidence exists that selective BLM's binding of RNA is entirely different than that of DNA. BLM binds RNA far more selectively with a preference to cleave RNAs at the junction between double- and single-stranded regions [5]. Also the anticodon of yeast $tRNA^{Phe}$ has been shown to be a sub-

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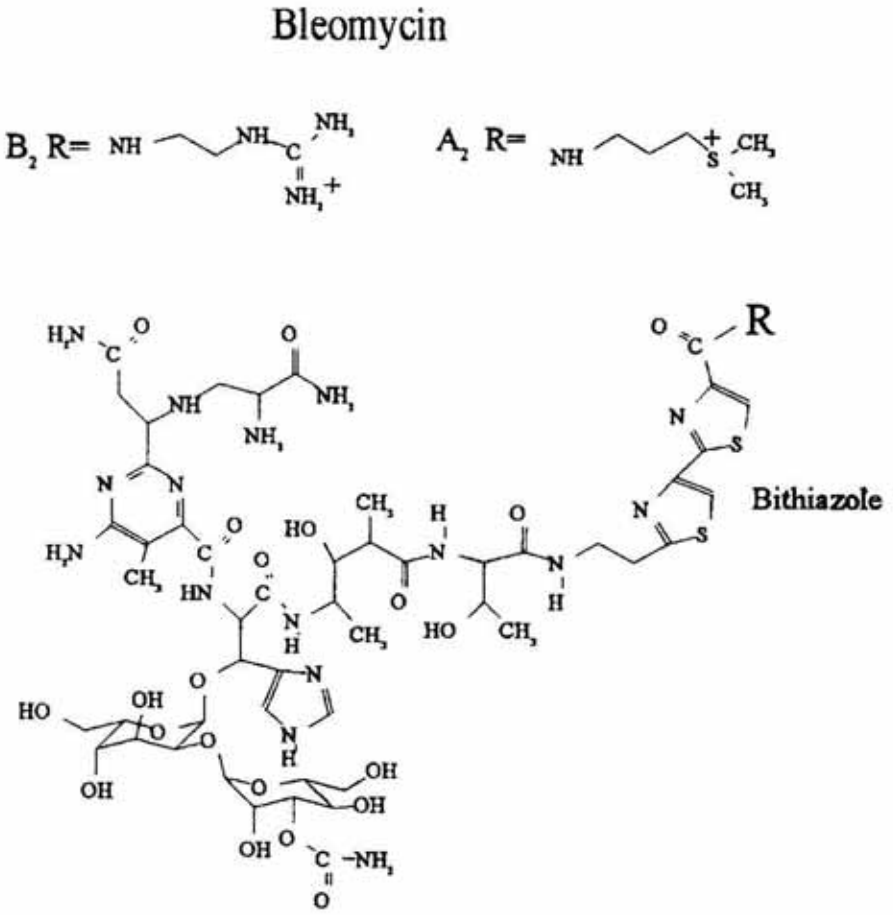
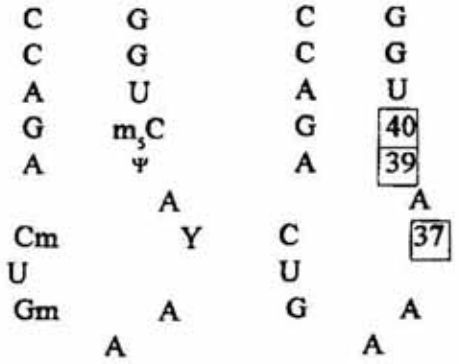
Abbreviations: AC, anticodon; BLM, bleomycin; CD, circular dichroism; $tRNA^{Phe}_{AC}$, oligonucleotides analogous to $tRNA^{Phe}$ anticodon.



Scheme 1. Bleomycin is a natural antibiotic that in commercial form contains two forms, A_2 and B_2, that vary only in the polyamine R group.

strate for Fe-BLM [6]. Like in the case of many RNA substrates, the Fe-BLM mediated cleavage of yeast $tRNA^{Phe}$ is sensitive to the presence of Mg^{2+} ions, which can completely inhibit cleavage of some RNAs substrates by Fe-BLM at ion concentrations above 1 mM [7]. The reasons for magnesium selectivity are unknown, but an understanding of them may be critical for the design of new therapeutics.

One factor that strongly influences Mg^{2+} binding to RNAs, and may influence BLM selectivity, are natural post-transcriptional modifications [8]. We have recently synthesized a series of RNA oligonucleotides analogous to the yeast $tRNA^{Phe}$ anticodon ($tRNA^{Phe}_{AC}$; Scheme 2) [9]. Using oligonucleotides that vary in the location and number of the five post-transcriptional modifications found in the anticodon of $tRNA^{Phe}$, differences in Mg^{2+} binding were detected by circular dichroism spectroscopy (CD) [10]. We now present preliminary results from fluorescence



Scheme 2. The anticodon stem loop of yeast $tRNA^{Phe}$ contains 17 nucleotides of which five are post-transcriptionally modified.

The modifications are as follows: 2′*O*-methylcytidine-32 and 2′*O*-methylguanosine-34 (Cm_{32} and Gm_{34}); wyeosine-37 (Y_{37}); pseudouridine-39 (ψ_{39}) and 5-methylcytidine-40 (m^5C_{40}).

and CD quantitating BLM's selective recognition of $tRNA^{Phe}_{AC}$ oligonucleotides. The same oligonucleotides were used to study the role magnesium ions play in BLM/RNA interaction.

METHODS

The oligonucleotides used in this study were synthesized on an ABI 394 DNA Synthesizer and purified by HPLC [9]. Bleomycin, purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and used without additional purification, was a mixture of A and B forms. In all experiments bleomycin was used in the absence of Fe^{2+} ions. Fluorescence data were collected with a Perkin-Elmer Model 650-10S fluorimeter. Excitation was provided by a xenon arc lamp at a wavelength of 300 nm with a slit width of 10 nm. Emission was monitored at a wavelength of 370 nm with a slit width of 10 nm. CD spectra were recorded using a 1 cm cell pathlength at 10°C on a JASCO 600 spectrometer scanning from 190 to 350 nm.

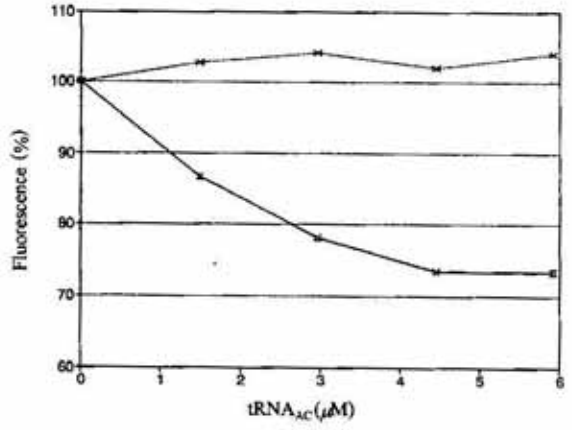


Figure 1. Effects of $tRNA^{Phe}_{AC}$ and $tRNA^{Glu}_{AC}$ on BLM's fluorescence.

The addition of $tRNA^{Phe}_{AC}$ quenches bleomycin's fluorescence (solid squares). In contrast, the addition of $tRNA^{Glu}_{AC}$ did not produce any quenching (dashed lines with X's). The concentration of BLM was 0.3 mM and concentrations of RNA were varied from 0 to 6 μM with all readings taken in triplicate.

RESULTS

The use of fluorescence spectroscopy is an effective method for monitoring complex formation between BLM and DNA [11]. We have monitored the changes in fluorescence of BLM on addition of RNA anticodon analogs. Addition of $tRNA^{Phe}_{AC}$ to BLM quenched the fluorescence, as shown in Fig. 1. In contrast, addition of a control RNA, a heptadecamer analog of $tRNA^{Glu}_{AC}$ anticodon, had no affect on the fluorescence of BLM. The quenching observed is similar to that observed for DNA binding. The fluorescence of BLM arises from the bithiazole rings and its quenching occurs when the rings are shielded in the complex. This shielding has been observed by NMR of a model system composed of Co-BLM bound to DNA [12]. The fluorescence changes observed during titration of BLM with $tRNA^{Phe}_{AC}$ indicated the formation of a complex in equilibrium with the two free components. From a molar ratio titration, the stoichiometry in the complex was determined to be 1:1 [10]. Results from titration experiments collected at multiple concentrations yielded the association constant $K_d = 1.1 \pm 0.2 \times 10^{-5}$ M. This value is quite similar to that determined by fluorescence for the affnity of BLM to cal thymus DNA [11].

The CD spectra of the RNA and BLM do not show an extensive overlaping (Fig. 2A). The addition of BLM to $tRNA^{Phe}_{AC}$ results in a CD spectrum different from the arithmetic sum of the individual components (Fig. 2B). This result is The CD spectra of the RNA and BLM do not show an extensive overlaping (Fig. 2A). The addition of BLM to $tRNA^{Phe}_{AC}$ results in a CD spectrum different from the arithmetic sum of the individual components (Fig. 2B). This result is characteristic when complex formation produces a conformational change in one or both of the components. The complex equilibrium was characterized, using CD, by titrations of $tRNA^{Phe}$ with BLM. The stoichiometry was determined to be 1:1 and the asso-

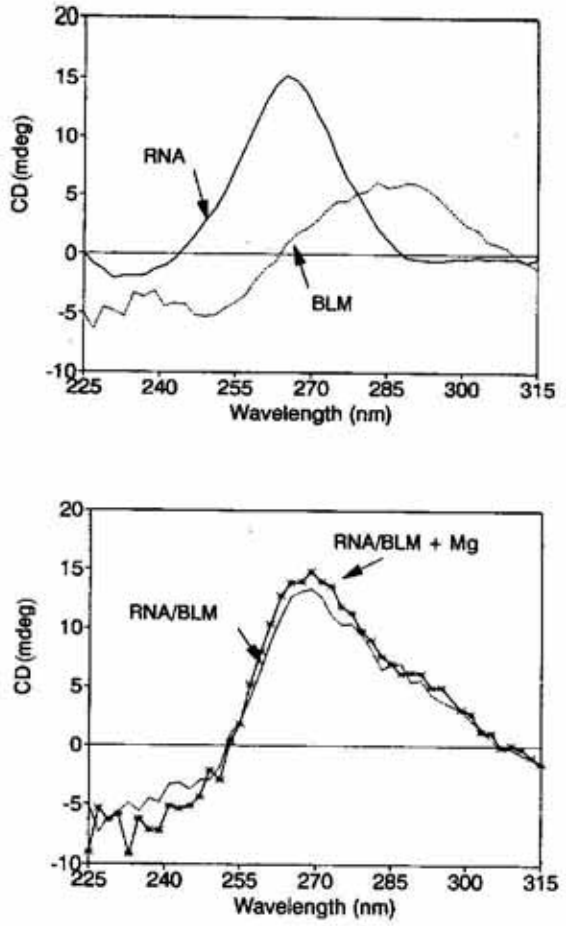


Figure 2. BLM/RNA complex formation followed by CD spectroscopy.

A) The individual CD spectra of $tRNA^{Phe}$ (solid) and BLM (dashed) do not have significant overlap. B) The observed spectra of BLM with RNA gives a spectrum (solid with triangles) that is not the same as the arithmetic sum of the individual components (dashed line).

ciation constant calculated as $K_d = 2.1 \pm 1 \times 10^{-5}$ M. This result is in agreement with the association constant determined by fluorescence spectroscopy.

The anticodon analogs were then used as a model system to investigate the role Mg^{2+} plays in BLM/RNA selectivity. At least two different hypotheses have been proposed to explain the effect of Mg^{2+} on interactions between RNA and BLM. One hypothesis proposes that RNA is not a true target for BLM, but that the drug weakly binds at a tight metal ion binding site that would not be available under physiological conditions [7]. For this hypothesis to be valid the Mg^{2+}/RNA binding constant should be lower than that for the BLM/RNA complex. The Mg^{2+}/RNA association constant for $tRNA^{Phe}_{AC}$, determined by CD methods, is 5×10^{-5} M (unpublished), thus higher than the BLM/RNA association constant. If this hypothesis is valid the BLM/RNA complex should be easily disrupted by magnesium. When 10 mM magnesium was added to a BLM/RNA complex no change was seen in the CD spectra (Fig. 3). Neither the higher Mg^{2+}/RNA association constant nor the inability of excess magnesium to disrupt the BLM/RNA support the hypothesis of BLM competing for ion tight binding sites.

According to an alternative hypothesis [5] BLM/RNA binding is conformationally dependent. The increased RNA selectivity in the presence of Mg^{2+} results from the reduction of favorable binding sites due to conformation changes induced by magnesium. According to this hypothesis BLM/RNA association constant would be lower than that of Mg^{2+}/RNA, as it was observed. Also favored by this hypothesis would be a slow equilibration of the BLM/RNA complex, in the presence of magnesium, due to an entropic barrier. In fact, slow equilibration was observed in CD spectra of a Mg/RNA complex when 60 mM BLM was added. No initial conversion of the BLM/RNA

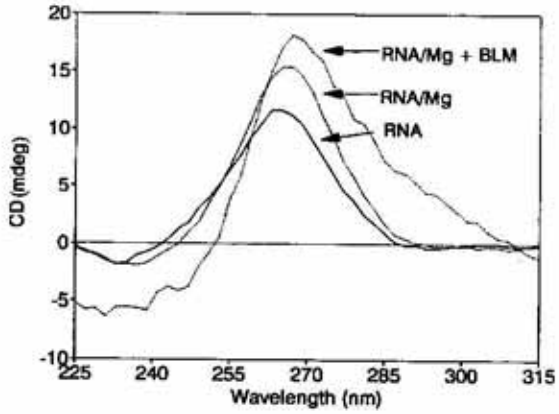


Figure 3. Effect of Mg^{2+} on BLM/RNA CD spectrum.

The CD spectrum of the BLM/RNA complex (dashed) is not altered by the addition of Mg^{2+} (solid with *'s).

complex was observed but a subsequent spectrum of the same sample collected after 5 days showed that a slow conversion to the BLM/RNA had occurred (Fig. 4).

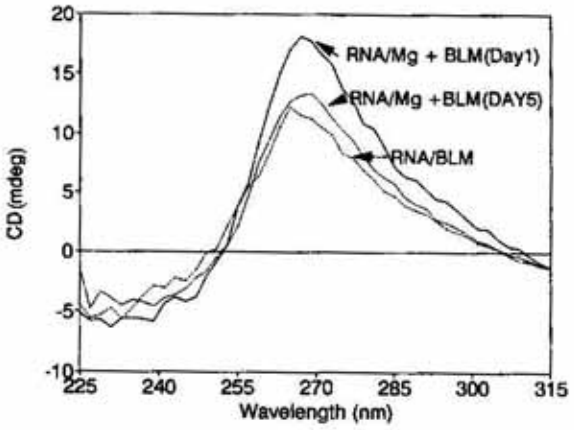


Figure 4. The BLM/RNA complex is in slow equilibrium in the presence of Mg^{2+}.

The CD spectrum the Mg^{2+}/RNA + BLM, 20 min post addition (solid line) is different from the spectrum of the BLM/RNA complex formed in the absence of Mg^{2+} (dashed line). After a five day incubation the CD spectrum (dotted line) has changed to resemble that of the B.

DISCUSSION

The therapeutic target of BLM is still unknown. The determination of an *in vitro* association constant for BLM/RNA comparable to that of BLM/DNA suggests that both RNA and DNA may be therapeutically relevant targets. The preliminary CD results support Mg^{2+}-induced conformational changes, rather than low affinity of BLM/RNA binding, as a source of BLM's selectivity. A selective conformational requirement of RNA substrates would require high affinity of RNA targets for therapeutic action. Beyond this example, the use of synthetic oligonucleotides containing natural modifications opens new approaches to investigate drug/RNA recognition. The flexibility afforded by automated chemical synthesis, provides a rational means to gaining access to the potential of modified RNAs that could serve as drug targets.

REFERENCES

1. Umezawa, H., Maeda, K., Takeuchi, T. & Okami, Y. (1966) New antibiotics, bleomycin A and B. *J. Antibiot.* **19**,200-209.

2. Blum, R.H., Carter, S.K. & Agre, K. (1973) A clinical review of bleomycin – a new antineoplastic agent. *Cancer* **31**, 903-914.

3. Stubbe, J. & Kozarich, J.W. (1987) Mechanisms of bleomycin-induced DNA degradation. *Chem. Rev.* **87**, 1107-1136.

4. Morgan, M.A. & Hecht, S.M. (1994) Iron (II) bleomycin-mediated degradation of a DNA-RNA heteroduplex. *Biochemistry* **33**, 10286-10293.

5. Holmes, C.E., Carter, B.J. & Hecht, S.M. (1993) Characterization of iron (II)·bleomycin- mediated RNA strand scission. *Biochemistry* **32**, 4293-4307.

6. Holmes, C.E. & Hecht, S.M. (1993) Fe · Bleomycin cleaves a transfer RNA precursor and its "transfer DNA" analog at the same major site. *J. Biol. Chem.* **268**, 25909-25913.

7. Huttenhofer, A., Hudson, S., Noller, H.F. & Mascharak, P.K. (1992) Cleavage of tRNA by Fe(II)-bleomycin. *J. Biol. Chem.* **267**, 24471-24475.

8. Agris, P.F. (1996) The importance of being modified: Roles of modified nucleosides and Mg^{2+} in RNA structure and function. *Prog. Nucleic Acid Res. Mol. Biol.* **53**, 79-129.

9. Agris, P.F., Malkiewicz, A., Krasewski, A., Everett, K., Nawrot, B., Sochacka, E., Jankowska, J. & Guenther, R. (1995) Site-selected introduction of modified purine and pyrimidine ribonucleosides into RNA by automated phosphoramidite chemistry. *Biochimie* **77**, 125-134.

10. Chen, Y., Sierzputowska-Gracz, H., Guenther, R., Everett, K. & Agris, P.F. (1993) 5-Methylcytidine is required for cooperative binding of Mg^{2+} and a conformational transition at the anticodon stem-loop of yeast phen-

ylalanine tRNA. *Biochemistry* **32**, 10249–10253.

11. Chien, M., Grollman, A.P. & Horwitz, S.B. (1977) Bleomycin–DNA interactions: Fluorescence and proton magnetic resonance studies. *Biochemistry* **16**, 3641–3647.

12. Xu, R.X., Nettesheim, D., Otvos, J.O. & Petering, D.H. (1994) NMR determination of the structures of peroxycobalt (III) bleomycin and cobalt (III) bleomycin, products of the aerobic oxidation of cobalt (II) bleomycin by dioxygen. *Biochemistry* **33**, 907–916.