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RNA-AMINOGLYCOSIDE ANTIBIOTIC INTERACTIONS: FLUORESCENCE DETECTION OF BINDING AND CONFORMATIONAL CHANGE

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Abstract: A hammerhead ribozyme has been labeled with a fluorescein reporter dye which enables the nucleic acid to detect binding of small organic compounds such as neomycin. The fluorescent changes are associated with conformational changes in the RNA and can be used to determine the binding modes of the drugs.

Aminoglycoside antibiotics are low molecular weight molecules that interact with a diverse range of RNAs.[1–7] The well known RNA-binding activity of these drugs is significant with respect to their antibactericidal properties and potential antiviral activities.[6–8] Although recent studies have revealed several important features of RNA binding by this class of molecules (reviewed in ref 9), many details of the binding processes remain unclear, in particular the multiple drug binding modes and conformational changes. Here, we report a sensitive fluorescent assay to examine the solution behavior of RNA conformation upon neomycin binding.

In general, aminoglycosides do not contain any distinguishable spectroscopic or reactive properties that allow convenient detection of their interactions with RNA. In contrast to previous studies that have employed dye-conjugated aminoglycosides,[10] we have fluorescently labeled the RNA. In this manner, the binding modes of non-labeled drugs can be identified without interference by the dye label. Furthermore, the drug concentration can be varied at a fixed RNA concentration, whereas in previous approaches the drug–dye concentration was typically fixed.[10] Although similar approaches were reported recently to study protein–RNA[11] or RNA–RNA binding,[12] we demonstrate here that fluorescently labeled RNAs can also detect the binding of low molecular weight organic ligands. To the best of our knowledge, this approach has not been used previously to monitor RNA–small molecule binding. We also present circular dichroism (CD) spectra to support the idea that fluorescent changes are associated with conformational changes in the RNA.

We have exploited the hammerhead ribozyme for our studies because of its known small-molecule binding and self-cleaving abilities.[5,13,14] The catalytic activity of this ribozyme is inhibited by certain ligands such as aminoglycoside antibiotics, tetracyclines, and drug analogues.[5,14–16] Thus, the hammerhead ribozyme offers an ideal model for testing our method because the kinetics of binding by a series of antibiotics have been reported and can be compared directly with the fluorescent data obtained in our binding studies. Furthermore, molecular details of this RNA have been elucidated through FRET, X-ray crystallography, and molecular modeling studies.[17–19] By employing fluorescent methods, we have revealed a novel mechanism for neomycin binding to the ribozyme. First, we will show the results of steady-state fluorescence and CD experiments, then discuss the potential of this RNA-labeling and fluorescence method as a tool that can be employed for future drug design.

The design of our fluorescent reporting system, shown in Figure 1A, is as follows. A 38-nucleotide ribozyme, R16, was prepared by in vitro transcription. The corresponding substrate, S16-F, was synthesized chemically with a fluorescein on its 5' end (details given in ref 20). As shown in Figure 1B, the dye is attached to the RNA through a six-carbon linker. The resulting hammerhead ribozyme complex (HH16-F) is altered in such a manner that it can now sensitively detect ligand interactions by changes in steady-state fluorescence.

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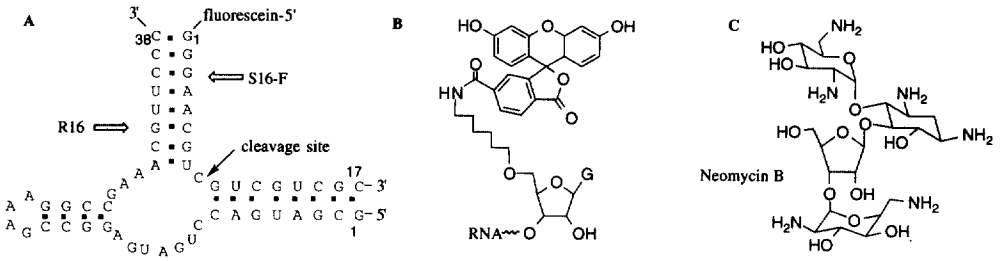

Figure 1. (A) The sequence and secondary structure of a 38-nucleotide hammerhead ribozyme (R16) complexed to a fluorescein-labeled (5' end) 17-nucleotide substrate (S16-F). The dye-labeled complex is referred to as HH16-F. (B) Details of the six-carbon linker between the fluorescein dye and RNA. (C) Structure of the aminoglycoside antibiotic, neomycin B.

Remote placement of the fluorescein moiety at the end of the substrate RNA does not have any inhibitory effects on the cleavage reaction,[21] but allows detection of long-range, drug-induced, conformational changes in HH16-F, as shown in Figure 2A. Binding of neomycin at concentrations of 0.1 μM to 10 mM leads to a quenching in the steady-state fluorescence of HH16-F (maximum of 84% quenching) and a corresponding 6 nm red shift. We have confirmed that neomycin is indeed binding to the complex, HH16-F and not free S16-F by gel analysis.[22] We believe that the quenching effect is the result of an alteration in the fluorescein environment upon neomycin binding, possibly through changes in stacking interactions of the dye with the ends of the RNA or restricted rotation due to interactions between helices. Studies are currently underway in our laboratory to address this issue. Meanwhile, we have confirmed that the effects on fluorescence are indirect, rather than from direct drug binding to the fluorescein, because a control fluorescein-labeled dimer (5' F-GG 3') shows negligible quenching upon addition of neomycin (<5% at 5 mM). Binding to S16-F in the absence of R16 demonstrates a different quenching behavior upon addition of neomycin, suggesting that the fluorescence method can also be used to study the interactions of neomycin and related aminoglycosides with single-stranded substrate molecules, which have been reported previously to have aminoglycoside binding activities.[15]

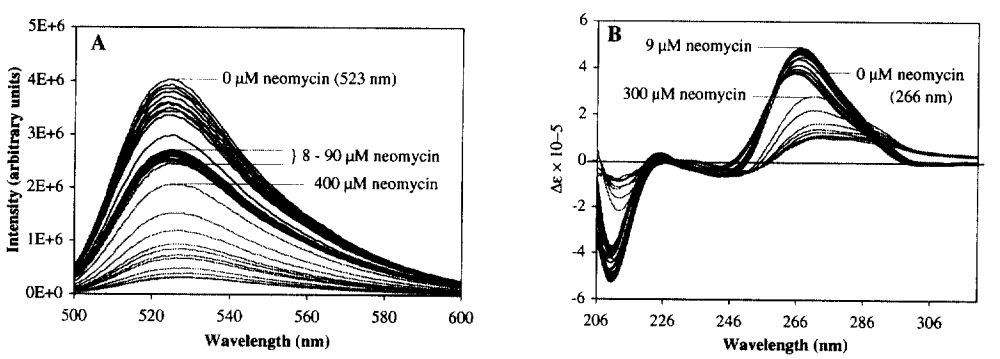

Figure 2. Spectral changes associated with neomycin binding to HH16-F. (A) The fluorescence emission spectra (recorded on a Spex Fluoromax) of 1 μM HH16-F (50 mM Tris-HCl, pH 7.3, 37 °C) upon addition of 0.1 μM to 10 mM neomycin B. A shift in the emission peak from 523 to 529 nm and quenching is observed (excitation wavelength = 490 nm). (B) An overlay of the CD spectra (recorded on a Jasco J-600 spectropolarimeter) of HH16-F under identical conditions as fluorescence measurements in (A) is shown.

The HH16-F fluorescence binding curve (Figure 2A) is reflective of several neomycin binding modes. A steady decrease in fluorescence is observed up to 7 μM added neomycin. Slight increases in fluorescence are observed between 8 and 90 μM, then further and significant decreases in fluorescence are observed from 90 μM to 5 mM neomycin. These data are suggestive of at least two distinct modes for neomycin binding to HH16-F. A plot of neomycin concentration versus relative fluorescence is shown in Figure 3. Figure 3A shows the data at

low drug concentration (0.1 to 5 μM) where the first binding mode is apparent. The apparent first-order fit is shown and gives a binding constant, K = 3.4 x 10^5 M^{-1}. This number is consistent with the reported K_i (13.5 μM) for neomycin inhibition of HH16,[5] and is also within an order of magnitude of the binding constants reported for other RNA–neomycin interactions.[23] A second binding mode is observed at higher neomycin concentrations (90 μM to 5 mM). The further increase in quenching above 90 μM is consistent with relative HH16 cleavage rates reported by Stage et al.[5] in which inhibition was ~7-fold greater at 1 mM compared to 100 μM neomycin. Figures 3B and 3C show the plots for relative fluorescence versus [neomycin] and [neomycin]^2, respectively. As can be seen from the curves, the [neomycin]^2 curve has a much better fit, suggesting that neomycin binds as a dimer, or has more than one binding site, at higher concentrations. The binding constant, K, obtained from the apparent second-order fit is 8 x 10^6 M^{-2}, whereas the first-order fit gives K = 4 x 10^3 M^{-1}. This result is consistent with reports by Wang and Tor in which a covalently-linked neomycin dimer had stronger inhibitory effects on HH16 cleavage at 1 and 10 μM concentrations compared to unlinked neomycin.[24] It is also consistent with earlier suggestions that aminoglycosides could have more than one recognition site within a single RNA.[1,5] Although previous work was suggestive of dimerization or multiple binding modes, our fluorescent results provide compelling evidence regarding the existence of such binding modes.

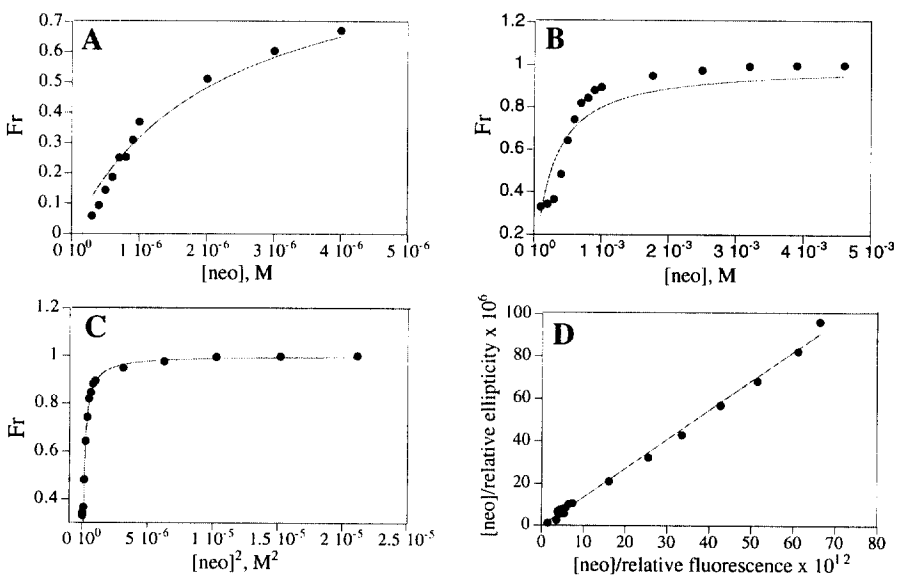

Figure 3. (A) A plot of relative fluorescence intensity (Fr) at 523 nm vs low [neomycin] (0.1 to 5 μM) and first-order curve fit. For high neomycin concentrations (0.09 to 5 mM), plots of Fr (523 nm) vs [neomycin] and first-ordercurve fit (B) or [neomycin]^2 and second-order curve fit (C) are shown. (D) A plot of [neomycin]/relative molar ellipticity vs [neomycin]/relative fluorescence.

In order to confirm that the drug-induced fluorescent changes of HH16-F are associated with conformational changes in the RNA, the binding of neomycin was monitored by circular dichroism (CD) spectroscopy. We titrated HH16-F (1 μM) with increasing concentrations of neomycin (0.1 μM to 1 mM) and observed changes in the CD spectra that were strongly correlated to those observed in the fluorescence titrations (Figure 2B). Low concentrations (0.1 to 7 μM) of the aminoglycoside led to an increase in the molar ellipticity of the positive peak (266 nm) with a concomitant red shift (2 nm). At concentrations >100 μM, neomycin binding to HH16-F caused a marked decrease in the molar ellipticity at 266 nm and a further red shift of 2 nm. The CD changes agree well with the fluorescent changes as shown in Figure 3D which correlates the relative fluorescence with relative molar ellipticity (a linear curve fit gives y = 1.37x - 0.36; R = 0.99). Together, the fluorescence and

CD experiments reveal that the hammerhead ribozyme undergoes a series of neomycin binding modes that are associated with distinct conformational changes.

In summary, we have shown that attachment of fluorescein to the hammerhead ribozyme allows detection and quantitative reporting of the binding of organic molecules that do not contain distinct spectroscopic markers. Fluorescence spectra of the ribozyme complex undergo specific changes upon neomycin binding which clearly reflect conformational changes as shown by direct comparison with CD spectra. Furthermore, multiple neomycin binding modes have been identified in this study, which were not observed previously. The hammerhead ribozyme serves as an excellent model system for this study because the drug-induced fluorescent changes can be compared directly with inhibition of ribozyme activity, which serves as an internal check for drug binding. We anticipate numerous applications of this method with other types of fluorescently tagged RNAs. For example, dye-labeled RNAs can be used to determine novel drug-binding modes, examine drug-induced conformational changes in the RNA, and allow for real-time monitoring of drug binding to RNA.

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References and Notes

1. Moazed, D.; Noller, H. F. *Nature* **1987**, *327*, 389.
2. Purohit, P.; Stern, S. *Nature* **1994**, *370*, 659.
3. von Ahsen, U.; Davies, J.; Schroeder, R. *Nature* **1991**, *353*, 368; von Ahsen, U.; Davies, J.; Schroeder, R. *J. Mol. Biol.* **1992**, *226*, 935.
5. Stage, T. K.; Hertel, K. J.; Uhlenbeck, O. C. *RNA* **1995**, *1*, 95.
6. Zapp, M. L.; Stern, S.; Green, M. R. *Cell* **1993**, *74*, 969.
7. Mei, H.-Y.; Galan, A. A.; Halim, N. S.; Mack, D. P.; Moreland, D. W.; Sanders, K. B.; Truong, H. N.; Czarnik, A. W. *Bioorg. Med. Chem. Lett.* **1995**, *5*, 2755.
8. Gale, E. F.; Cundliffe, E.; Reynolds, P. E.; Richmond, M. H.; Waring, M. J. *The Molecular Basis of Antibiotic Action*; John Wiley & Sons: New York, 1972.
9. Tor, Y.; Hermann, T.; Westhof, E. *Chem. Biol.* **1998**, *5*, R277.
10. Hamasaki, K.; Killian, J.; Cho, J.; Rando, R. R. *Biochemistry* **1998**, *37*, 656.
11. Potyrailo, R. A.; Conrad, R. C.; Ellington, A. D.; Hieftje, G. M. *Anal. Chem.* **1998**, *70*, 3419.
12. Walter, N. G.; Burke, J. M. *RNA* **1997**, *3*, 392.
13. Hertel, K. J.; Herschlag, D.; Uhlenbeck, O. C. *Biochemistry* **1994**, *33*, 3374.
14. Clouet-d'Orval, B.; Stage, T. K.; Uhlenbeck, O. C. *Biochemistry* **1995**, *34*, 11186.
15. Murray, J. B.; Arnold, J. R. P. *Biochem. J.* **1996**, *317*, 855.
16. Wang, H.; Tor, Y. *J. Am. Chem. Soc.* **1997**, *119*, 8734.
17. Bassi, G. S.; Møllegaard, N.-E.; Murchie, A. I. H.; von Kitzing, E.; Lilley, D. M. J. *Struct. Biol.* **1995**, *2*, 45; Bassi, G. S.; Murchie, A. I. H.; Walter, F.; Clegg, R. M.; Lilley, D. M. J. *EMBO J.* **1997**, *16*, 7481; Menger, M.; Tuschl, T.; Eckstein, F.; Porschke, D. *Biochemistry* **1996**, *35*, 14710.
18. Pley, H. W.; Flaherty, K. M.; McKay, D. B. *Nature* **1994**, *372*, 68; Scott, W. G.; Finch, J. T.; Klug, A. *Cell* **1995**, *81*, 991.
19. Hermann, T.; Westhof, E. *J. Mol. Biol.* **1998**, *276*, 903.
20. The synthesis was carried out with Fpmp RNA and 5'-fluorescein phosphoramidites (Cruachem). The RNA was removed from the support with standard conditions (conc. NH_4OH, 55 °C, 12 h) and 2'-*O*-deprotected under mild acidic conditions.
21. The kinetics of cleavage of ^{32}P 3'-end-labeled S16-F and S16 (no fluorescein) by R16 were compared directly using conditions reported previously.[5] The HH16 cleavage reaction is not inhibited by the presence of the fluorescein moiety, and the ability of neomycin to inhibit the reaction is not affected (data not shown).
22. HH16-F in the presence or absence of neomycin (up to 10 mM) (under conditions as stated in Figure 2) was examined on a 4% non-denaturing polyacrylamide gel. There was no evidence of either free R16 or S16-F on the gel by visualizing the fluorescein fluorescence or ethidium bromide staining, whereas the HH16-F band was clearly visible and shifted slightly in the presence of neomycin (data not shown).
23. In other studies, a variety of salt conditions and different RNAs were employed.[10,25] Here, buffer conditions identical to those reported by Stage et al.[5] for determination of neomycin inhibition of HH16 were used.
24. Wang, H.; Tor, Y. *Bioorg. Med. Chem. Lett.* **1997**, *7*, 1951.
25. Wong, C.-H.; Hendrix, M.; Priestley, E. S.; Greenberg, W. A. *Chem. Biol.* **1998**, *5*, 397.