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Interactions of the antibiotics neomycin B and chlortetracycline with the hammerhead ribozyme as studied by Zn^{2+}-dependent RNA cleavage

Emily J. Borda and Snorri Th. Sigurdsson*

Department of Chemistry, University of Washington, Seattle, WA 98195-1700, USA

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Abstract—We have investigated the interactions of two antibiotics, neomycin B and chlortetracycline (CTC), with the hammerhead ribozyme using two Zn^{2+} cleavage sites at U4 and A9 in its catalytic core. CTC-dependent inhibition of Zn^{2+} cleavage was observed in all cases. In contrast, we unexpectedly observed acceleration of A9 cleavage by neomycin under low ionic strength conditions similar to those used to study inhibition of hammerhead substrate cleavage by this antibiotic. This result provides evidence that the inhibitory mechanism of neomycin does not include competition with the metal ion bound to the A9/G10.1 metal-ion binding site, as previously proposed. Under high ionic strength conditions, optimized for Zn^{2+}-dependent cleavage, we observed neomycin-dependent inhibition of cleavage at both A9 and U4. The ability of neomycin to both inhibit and accelerate Zn^{2+} cleavage suggests that there is either more than one neomycin binding site or multiple binding modes at a single site in the hammerhead ribozyme. Furthermore, the accessibilities and/or affinities of disparate neomycin binding sites or binding modes are dependent on the ionic strength and the pH of the medium.

1. Introduction

The hammerhead ribozyme is a catalytic RNA motif that facilitates sequence-specific cleavage in the sugar-phosphate backbone of an RNA substrate (for review, see ref 1). Divalent metal ions have been implicated both directly[2,3] and indirectly[4,5] in this ribozyme's catalytic mechanism. We have recently used Zn^{2+}-catalyzed cleavage to further our understanding of the roles of such metal ions in the hammerhead ribozyme.[6] Figure 1A depicts two Zn^{2+}-dependent cleavage sites within the hammerhead-substrate complex. A Zn^{2+} ion inhabiting the well-characterized metal-ion binding site between nucleotides A9 and G10.1[7–10] catalyzes cleavage between nucleotides G8 and A9 and does not require the active tertiary structure for cleavage.[11] The other site, between C3 and U4, must be preceded by A9 cleavage and, in contrast to A9 cleavage, only occurs at relatively high pH (>7.9).[6] Both of these properties of U4 cleavage, along with a requirement for a non-cleavable substrate strand, suggest U4 cleavage is highly dependent upon formation of a specific tertiary structure, which is different from the active conformation. Consequently, the location of the Zn^{2+} ion causing cleavage at this site is heretofore unknown.

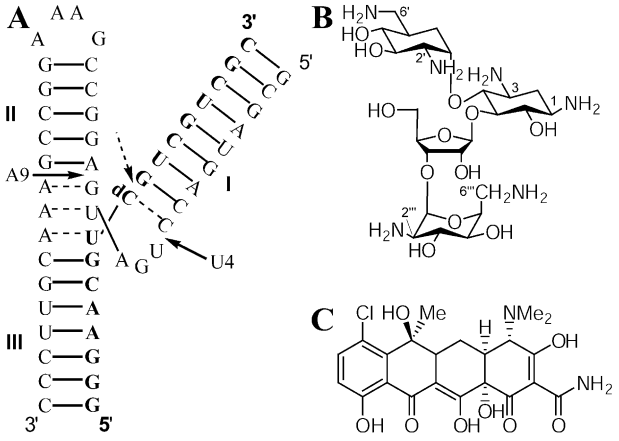

Figure 1. (A) The hammerhead ribozyme (HH16 construct), with substrate strand shown in bold. The substrate cleavage site is shown with a dashed arrow. The Zn^{2+}-catalyzed cleavage sites U4 and A9 are shown with bold arrows; (B) Structure of neomycin B, with numeric positions of amino groups indicated on the carbons to which they are attached; (C) Structure of chlortetracycline.

Keywords: Metal ion-dependent RNA cleavage; Neomycin B; Chlortetracycline; Zinc.
* Corresponding author. Tel.: +1-206-616-8276; fax: +1-206-685-8665; e-mail: sigurdsson@chem.washington.edu

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Here we describe the use of these two Zn^{2+}-catalyzed cleavage sites to study the mechanism of inhibition of two antibiotics, the aminoglycoside-class antibiotic neomycin B (Fig. 1B) and the tetracycline-class antibiotic chlortetracycline (CTC) (Fig. 1C). Both of these antibiotics have been shown to inhibit hammerhead ribozyme substrate cleavage activity.[12–14] Based on molecular modeling studies, the mechanism of inhibition by neomycin B has been suggested to occur through competition of its protonated amino groups with essential divalent metal ions, including the one at A9/G10.1.[15] CTC also inhibits hammerhead ribozyme catalysis; however, acceleration of substrate cleavage has been observed at low concentrations of this antibiotic.[14] We have incubated neomycin B and CTC with the hammerhead ribozyme under conditions used to study inhibition of substrate cleavage (low ionic strength) and conditions optimized for Zn^{2+}-dependent cleavage (high ionic strength) and monitored formation of the A9 and U4 cleavage products. While CTC-dependent inhibition of A9 and U4 cleavage was observed in all cases, neomycin exhibited both inhibition and acceleration of A9 cleavage. Acceleration was unexpectedly observed under the low ionic strength conditions, providing evidence against competitive inhibition by neomycin at the A9/G10.1 metal ion binding site as an inhibitory mechanism of substrate cleavage. The fact that both neomycin-dependent inhibition and acceleration of A9 cleavage were observed suggests that there exist a number of binding sites, or different binding modes for a single binding site, in the hammerhead ribozyme for this antibiotic.

2. Results

2.1. Antibiotic titrations

The well-characterized HH16 hammerhead ribozyme construct[16] was used in these studies. To prevent cleavage of the substrate, the ribozyme was complexed with a substrate strand containing a 2′-deoxycytosine at the cleavage site (Fig. 1A). This complex was incubated with varied concentrations of antibiotic under the relatively low ionic strength conditions in which inhibition of substrate cleavage was previously reported.[13,14] Because the hammerhead has been shown to be catalytically proficient under these conditions,[13,14] we can assume it is in its native conformation. It should be noted that we cannot comment on the effects of the antibiotics on U4 cleavage under these conditions because the U4 product is only observed above pH 7.8.[6]

In contrast to CTC, which showed up to 46% inhibition of A9 cleavage, neomycin accelerated cleavage at A9 (Fig. 2). To rule out divalent metal ion contamination from the neomycin solution as the cause of acceleration, Zn^{2+}-dependent cleavage at 100 μM neomycin was monitored in the presence of 0–1 mM EDTA, which was added to the neomycin before coming in contact with the rest of the solution. No significant fluctuation in A9 cleavage was observed below 50 μM EDTA. At and above this concentration, A9 cleavage started to drop off dramatically due to chelation of the Zn^{2+}, which was present at 100 μM (data not shown). An alternate explanation for neomycin-induced acceleration of A9 cleavage is that exchange between Mg^{2+} and Zn^{2+} could be mediated by neomycin. This was ruled out by demonstrating that annealing the ribozyme-substrate complex in the presence of both Zn^{2+} and Mg^{2+} produced results similar to those obtained when the complex was annealed first in the presence of Mg^{2+} and then allowed to complex with Zn^{2+} (data not shown). Finally, similar results obtained in the presence of a Tris buffer, rather than the MOPS buffer used in the analogous substrate cleavage study,[13] suggested that the counterion in the MOPS buffer was not sequestering Zn^{2+} ions. Interestingly, we also saw activation of A9

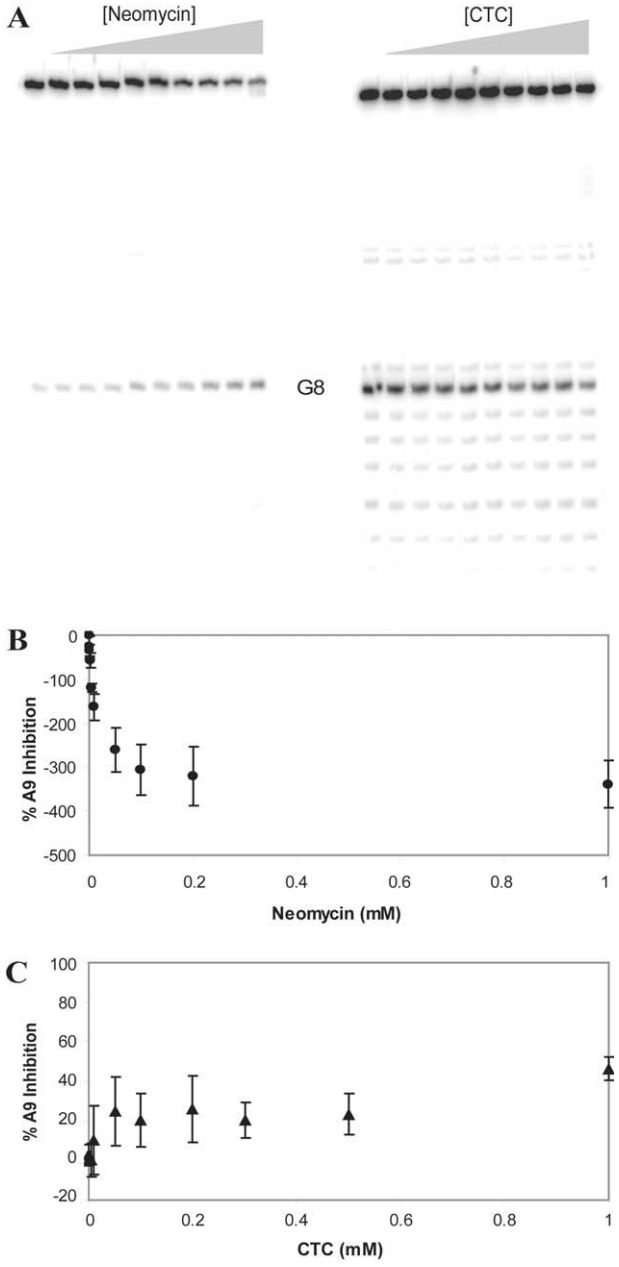

Figure 2. (A) DPAGE analysis of neomycin B and CTC titrations under native conditions (low ionic strength). Graphical analysis of this data for (B) neomycin B and (C) CTC.

cleavage with increasing concentrations of Na^+ and spermine, both of which are thought to take on the role of secondary and tertiary structure stabilization of the hammerhead ribozyme[2] (data not shown). In fact, the extent of A9 cleavage after 24 h under low ionic strength conditions in the presence of 100 μM neomycin (9.8%) was similar to the extent of cleavage when the neomycin was substituted for 500 mM NaCl (7.8%, data not shown).

In order to better understand the interactions of these antibiotics with the hammerhead ribozyme, we performed antibiotic titrations using conditions that we had previously optimized for Zn^{2+} cleavage.[6] These conditions differ from those in which inhibition of substrate cleavage was observed,[13,14] in that the ionic strength and pH were higher. In order to study A9 cleavage under these conditions without complicating our results with concurrent U4 cleavage, we stopped incubation of the reaction mixtures after 3 h, at which time U4 cleavage products had not formed to an appreciable extent.[6] In these experiments, we observed up to 81% inhibition of A9 cleavage by neomycin and 77% inhibition of A9 cleavage by CTC (1 mM both antibiotics, Fig. 3). Study of U4 cleavage required precleavage at A9 using a low concentration of Zn^{2+}. After most of the ribozyme had cleaved at A9, the concentration of Zn^{2+} was increased to initiate U4 cleavage. Inhibition of U4 cleavage was observed except in the presence of 100 μM neomycin, in which case 9.4% acceleration was observed. Complete U4 inhibition was observed at 1 mM neomycin and 800 μM CTC (Fig. 3).

2.2. pH titrations

When the hammerhead ribozyme-substrate complex was incubated in the presence of both antibiotics in concentrations known to inhibit substrate cleavage (low ionic strength),[13,14] under different pH conditions (6.5–8.6), A9 cleavage remained relatively constant except for a marked acceleration in the presence of 10 μM neomycin at pH 7.1 (Fig. 4A). Acceleration (161%) at this pH was over four-fold higher than acceleration at any other pH in the presence of neomycin. Under high ionic strength conditions, however, an increase in inhibition of A9 cleavage by neomycin was observed with increasing pH until pH 8.3, after which a decrease in inhibition was observed (Fig. 4B). The inhibition activity of CTC decreased with increasing pH in nearly a linear fashion, from 81% at pH 7.2 to 15% at pH 8.7 at high ionic strength. It should be noted, however, that decreased reproducibility above pH 8.3 under these conditions, possibly due to some precipitation of $Zn(OH)_2$[17] or partial denaturing of the RNA as the pH approaches the pK_a's of the imino protons, makes the prediction of trends in this region of the pH profiles difficult.

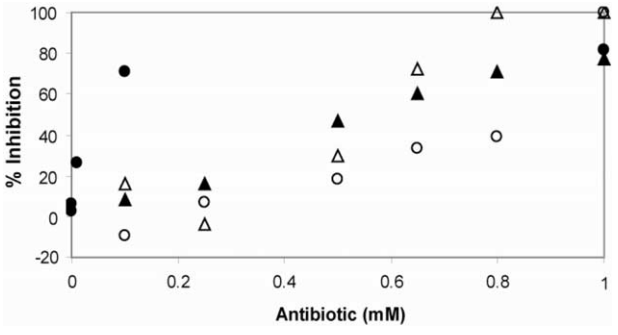

Figure 3. Effects of neomycin (circles) and CTC (triangles) on A9 cleavage (filled) and U4 cleavage (hollow) under high ionic strength conditions.

3. Discussion

3.1. Neomycin

Two different activities of neomycin were observed from these experiments. Under high ionic strength conditions, neomycin-dependent inhibition of A9 cleavage was observed (Fig. 3). However, under conditions similar to those in which inhibition of substrate cleavage by neomycin was observed,[13] formation of A9 cleavage product was unexpectedly accelerated by the antibiotic (Fig. 2B). Since the A9/G10.1 site is where the Zn^{2+} ion causing A9 cleavage is located,[11] this result suggests that competition of neomycin with the metal ion bound to the A9/G10.1 site is not part of neomycin's mechanism of substrate cleavage inhibition, as previously suggested.[15]

This dual action of neomycin could be explained by different modes of binding by neomycin to the same site

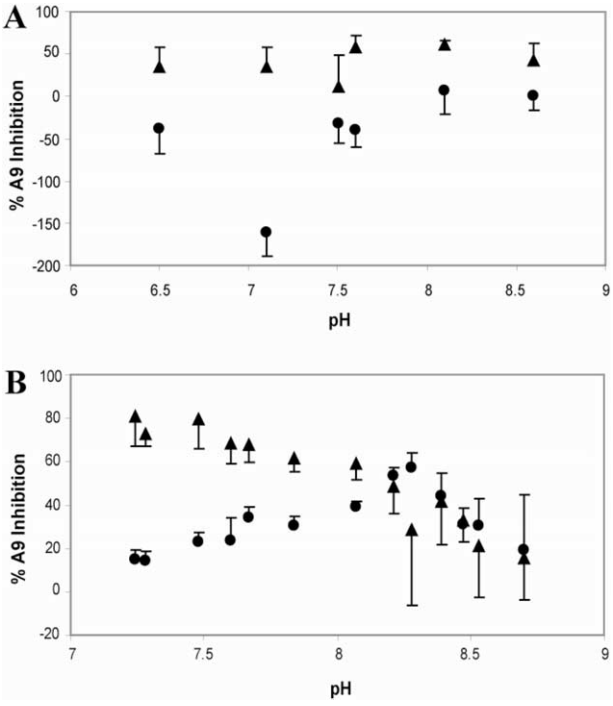

Figure 4. pH titrations with 10 μM neomycin (circles) and 500 μM CTC (triangles) under (A) native conditions (low ionic strength) and (B) high ionic strength conditions. Error bars apply to both sides of data points but are shown only on one side in order to prevent overlap between data sets.

under different conditions or the presence of multiple neomycin binding sites, some of which accelerate A9 cleavage when inhabited by the antibiotic, and one or more which cause inhibition of A9 cleavage when bound to neomycin. The possibility of more than one neomycin binding site has been suggested based on molecular modeling[18] and experiments in which conjugation of two neomycin molecules increased antibiotic affinity towards the hammerhead ribozyme.[19] It has also been shown by electron paramagnetic resonance (EPR) spectroscopy that ionic strength affects relative affinities of different sites toward cations: Four Mn^{2+} ions were bound to specific sites in the hammerhead ribozyme in the presence of 0.1 M NaCl whereas only one Mn^{2+} ion was bound (to the A9/G10.1 metal-ion binding site) in 1 M NaCl.[20] Ionic strength has also been shown to affect inhibition of substrate cleavage by neomycin; inhibition was dramatically reduced at NaCl concentrations at or above 500 mM.[21]

Our data suggest a model in which neomycin competes for the essential divalent metal ion in the A9/G10.1 binding site and thereby inhibits Zn^{2+}-dependent A9 cleavage at high ionic strength. This model is corroborated by an EPR spectroscopy study that showed neomycin displacement of a Mn^{2+} ion bound to the A9/G10.1 metal ion binding site at high ionic strength (1 M NaCl).[22] However, under native conditions (low ionic strength), it appears that neomycin prefers binding to a site that accelerates A9 cleavage. Alternatively, at low ionic strength, neomycin may be binding non-specifically to the ribozyme and altering its tertiary structure in a way that makes Zn^{2+}-catalyzed cleavage at A9 more favorable. The acceleration of RNA cleavage in the presence of aminoglycosides has also been observed for the hairpin ribozyme in the absence of metal ions.[23]

Neomycin-dependent inhibition of U4 cleavage was also observed under high ionic strength conditions (Fig. 3). U4 is located in the conserved U-turn within the catalytic core of the hammerhead ribozyme, close to where catalysis occurs. The observation that neomycin inhibits Zn^{2+} cleavage at this site indicates that the antibiotic binds in the vicinity of this important region at high ionic strength. However, because A9 cleavage is required for U4 product formation,[6] the ribozyme's active tertiary structure is likely perturbed, which could affect the location of neomycin binding site(s). The effects of neomycin on U4 cleavage under conditions used to study neomycin inhibition of substrate cleavage could not be investigated since U4 cleavage only occurs above pH 7.8.

Under native conditions (low ionic strength), an unusual pH profile is observed for neomycin. A gradual increase in A9 inhibition (decrease in acceleration) by neomycin is observed, except at pH 7.1 (Fig. 4A), where a dramatic increase in A9 cleavage was observed. We do not have a good explanation for these data, which imply a change in ionization state for either the ribozyme or the neomycin at this pH that would affect neomycin binding. For ionization of neomycin, the amine in position three has the lowest pK_a of 6.9.[24] It is plausible that protonation of this amine could cause acceleration at pH 7.1, if it were not for the extent of cleavage at pH 6.5 being similar to that observed at pH 7.5. In fact, the amount of protonated amine in position three ranges from 20-70% in the pH range 6.5-7.5, and therefore it is unlikely that the change in protonation state of neomycin in this pH range is the cause of the observed pH profile.

At high ionic strength, inhibition of A9 cleavage by neomycin increases with increasing pH until pH 8.3 (Fig. 4B). This is contrary to the expectation that the antibiotic should be more effective at inhibiting A9 activity through exchange at the A9/G10.1 binding site if more of its amine groups are protonated, because of increased electrostatic attraction of the antibiotic to the negatively charged RNA. We can only speculate that at high pH or ionic strength, the hammerhead's conformation may change or the affinity of the A9/G10.1 site for Zn^{2+} may decrease, facilitating exchange between Zn^{2+} and the antibiotic. Also, changes in the locations of positive charges in neomycin itself could affect which binding site(s) are preferred in ways that are difficult to predict because of potential cooperative effects between the charged groups.

3.2. Chlortetracycline

Murray and co-workers described acceleration of substrate cleavage at low concentrations but inhibition at high concentrations of CTC,[14] whereas we observed only inhibition of A9 cleavage in CTC titration experiments (Fig. 3). U4 inhibition was also observed, providing evidence for the presence of a binding site for CTC in the vicinity of the U-turn of the ribozyme.

The pH titrations with CTC revealed interesting aspects of the interaction of this antibiotic with the hammerhead ribozyme. In contrast to neomycin, which exists in multiple protonation states within the pH range studied in this report, the only functional group on CTC that has a pK_a within this pH range is the phenolic proton, with a pK_a of 7.44.[25] The protonated dimethyl amine group has a pK_a of 9.27 and the tricarbonylmethane proton (shown as enol in Fig. 1C) has a pK_a of 3.30. Thus, between pH 4 and 7, CTC is predominantly a neutral zwitterion. We would therefore expect a marked change in cleavage activity around pH 7.5 if ionic interactions exclusively governed CTC's effect on Zn^{2+} cleavage. However, CTC's pH profile under high ionic strength conditions shows that the antibiotic's ability to inhibit A9 cleavage increases gradually with decreasing pH (Fig. 4B). This result suggests that ionic interactions are responsible in part for CTC's effects on A9 cleavage at high ionic strength but because of the lack of dramatic change in inhibition around pH 7.5, other types of intermolecular forces such as hydrogen bonding and hydrophobic interactions are also important. Little or no pH dependency was observed under the low ionic strength conditions similar to those used to detect inhibition of hammerhead substrate cleavage by this antibiotic (Fig. 4A)

4. Conclusion

The most striking feature of these results is the dual action (inhibition and acceleration of Zn^{2+}-specific cleavage) exhibited by neomycin, which is highly dependent upon the aqueous environment of the ribozyme, specifically ionic strength and pH. There appears to be either more than one binding site for neomycin in the ribozyme or a single site with different modes of neomycin binding. Acceleration or inhibition of Zn^{2+} cleavage depends on which of these sites bind neomycin or in which mode the antibiotic binds under the experimental conditions. We have seen, for example, that neomycin accelerates A9 cleavage under native conditions (low ionic strength) and neutral pH. This particular result is notable as it is evidence against direct competition with the essential metal ion residing in the A9/G10.1 binding site, which has previously been proposed as a mechanism of substrate cleavage inhibition by neomycin.[15] It should be noted that the substrate-cleavage inhibition mechanism of neomycin could involve displacement of other essential divalent metal ions in the ribozyme, for example those in closer proximity to the substrate cleavage site.

Although we have described potential models of inhibition and activation of Zn^{2+} cleavage by these antibiotics in terms of ionic exchange mechanisms, it is possible that neomycin and/or CTC could also affect Zn^{2+}-dependent cleavage by altering the ribozyme's global conformation through more general electrostatic or non-ionic interactions. Regardless of the specific mechanisms involved in these interactions, our observations show that pH and ionic strength are imperative to keep in mind when targeting RNA with antibiotics in vivo.

5. Experimental

5.1. General

Oligoribonucleotides were purchased from Dharmacon Research, Inc., deprotected, 5′-^{32}P-labeled and purified as previously described.[11] Radiolabeled oligoribonucleotides were present only in trace amounts in any given sample. Analyses of cleavage experiments were performed on 20% denaturing polyacrylamide gels containing 7 M urea, 90 mM Tris, 90 mM boric acid and 1 mM Na_2EDTA. Cleavage experiments were analyzed on 0.4-mm sequencing gels at 3000 V for 2.5 h. The bands were visualized by phosphorimaging (Molecular Dynamics 400A PhosphorImager) and analyzed using Molecular Dynamics ImageQuant (version 5.1) software. Effects of the antibiotics on Zn^{2+} cleavage were quantified by the formula, % Inhibition $= (1–P_0/P_x)\times 100$, where P is the % A9 or U4 cleavage in the absence (P_0) or presence (P_x) of antibiotic, as determined by phosphorimagery. Error bars on graphs represent standard deviations between 3–4 independent trials.

5.2. Effects of antibiotics on A9 cleavage: Low ionic strength

Reaction mixtures containing 1 μM 5′-^{32}P labeled ribozyme (HH16), 1.1 μM noncleavable substrate (HH16S-dC17), 20 mM $MgCl_2$, 0.1 M buffer (MES-HCl pH 6.1, PIPES-HCl pH 6.5, MOPS-HCl pH 7.1, Tris–HCl pH 7.6, HEPES-HCl pH 7.6 or 8.1 or TAPS-HCl pH 8.6) were incubated at 70 °C for 2 min then allowed to equilibrate to room temperature to anneal the ribozyme and substrate (5 μL total volume). H_2O or neomycin B (1, 4, 8, 20, 40, 200, 400, 800 or 4000 μM, 2.5 μL) was added to the reaction mixtures which were allowed to equilibrate at room temperature for 15 min to allow the antibiotic to bind to the RNA complex, after which 400 μM $Zn(OAc)_2$ (2.5 μL) was added to each solution. Final concentrations (10 μL): 500 nM HH16, 550 nM HH16S-dC17, 50 mM buffer, 10 mM $MgCl_2$, 100 μM $Zn(OAc)_2$ and 0, 0.25, 1, 2, 5, 10, 50, 100, 200 or 1000 μM neomycin B. The reaction mixtures were incubated at 37 °C for 24 h (pH titrations) or 70 h (neomycin titrations) then combined with stop-mix (1:24 10 mM Na_2EDTA:formamide, 10 μL) and analyzed by denaturing polyacrylamide gel electrophoresis (DPAGE). Neomycin titrations were performed at a constant pH of 7.1 (MOPS-HCl) and pH titrations were carried out with 0 or 10 μM neomycin. The same procedure was used for CTC, with the following exceptions: The final concentrations of HH16 and HH16S-dC17 (1 and 1.1 μM, respectively) were double those used in the reactions involving neomycin and CTC titrations were performed at a constant pH of 7.6 (Tris–HCl). The final concentrations of CTC used in CTC titrations were 0, 1, 5, 10, 50, 100, 200, 300, 500 and 1000 μM, and pH titrations were carried out with 0 or 500 μM CTC.

5.3. Effects of antibiotics on A9 cleavage: High ionic strength

Reaction mixtures containing 500 nM 5′-^{32}P labeled HH16, 4 μM HH16S-dC17, 1 M NaCl, 20 μM Na_2EDTA and 100 mM Tris–HCl, pH 7.24, 7.28, 7.48, 7.60, 7.67, 7.84, 8.07, 8.21, 8.28, 8.39, 8.47, 8.53, 8.70 or 8.94 were annealed as above (5 μL total volume). H_2O, neomycin B (0.4, 4, 40, 400 μM, 4 or 40 mM) or CTC (0.1, 0.25, 0.5, 0.65, 0.8 or 1 mM) (2.5 μL) was added to the reaction mixtures. The solutions were allowed to equilibrate at room temperature for 15 min then combined with 800 μM $Zn(OAc)_2$ (2.5 μL). Final concentrations (10 μL): 250 nM HH16, 2 μM HH16S-dC17, 10 μM Na_2EDTA, 500 mM NaCl, 50 mM Tris–HCl, 200 μM $Zn(OAc)_2$, 0, 0.1, 1, 10, 100 μM, 1 mM or 10 mM neomycin B, or 0.1, 0.25, 0.5, 0.65, 0.8 or 1 mM CTC. Reaction mixtures were incubated at 37 °C for 3 h then combined with stop-mix (10 μL) and analyzed by DPAGE. Antibiotic titrations were performed at a constant pH of 8.5 and pH titrations were carried out with no antibiotic, 10 μM neomycin or 500 μM CTC.

5.4. Effects of antibiotics on U4 cleavage: High ionic strength

Radiolabeled HH16 (5′-^{32}P, 10 μM, 26.25 μL) was combined with 10 μM HH16S-dC17 (210 μL), pH 8.5 buffer (250 mM Tris–HCl, 2.5 M NaCl, 50 μM Na_2EDTA, 210 μL) and H_2O (46 μL). The solution was annealed as above, combined with 800 μM $Zn(OAc)_2$

(32.75 μL), and incubated at 37 °C until >90% of the ribozyme had been cleaved at A9 (10 d, by DPAGE). The reaction mixture was divided into 5-μL aliquots, which were combined with H_2O, neomycin B (0.4, 4, 40, 400 μM, 4 or 40 mM) or CTC (0.4, 1, 2, 2.6, 3.2 or 4 mM, 2.5 μL). The solutions were allowed to equilibrate at room temperature for 15 min, then combined with 700 μM $Zn(OAc)_2$ (2.5 μL). Final concentrations (10μL): 250 nM A9 cleavage product, 2 μM substrate, 10 μM Na_2EDTA, 500 mM NaCl, 50 mM Tris–HCl, 200 μM $Zn(OAc)_2$, 0, 0.1, 1, 10, 100 μM, 1 or 10 mM neomycin B, or 0.1, 0.25, 0.5, 0.65, 0.8 or 1 mM CTC. Reaction mixtures were incubated at 37 °C for 24 h then combined with stop-mix (10 μL) and analyzed by DPAGE.

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