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Binding of an aminoacridine derivative to a GAAA RNA tetraloop

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Abstract—RNA tetraloops are common secondary structural motifs in many RNAs, especially ribosomal RNAs. There are few studies of small molecule recognition of RNA tetraloops although tetraloops are known to interact with RNA receptors and proteins, and to form nucleation sites for RNA folding. In this paper, we investigate the binding of neomycin, kanamycin, 2,4-diaminoquinazoline, quinacrine, and an aminoacridine derivative (AD1) to a GAAA tetraloop using fluorescence spectroscopy. We have found that AD1 and quinacrine bind to the GAAA tetraloop with the highest affinity of the molecules examined. The equilibrium dissociation constant of the AD1–GAAA tetraloop complex was determined to be 1.6 μM. RNase I and lead acetate footprinting experiments suggested that AD1 binds to the junction between the loop and stem of the GAAA tetraloop.

RNA is essential in gene replication, expression, and processing and is important in the life cycles of pathogens.[1] Therefore, small molecules that could bind to RNA and inhibit its interactions with proteins and other RNAs would be of great utility.[2–6] RNAs fold into diverse structures that are important for the different cellular functions of RNA in cells. The diversity of these structures allows recognition by small molecules to be mediated by structure-specific as well as sequence-specific interactions. High resolution structures of large RNAs have identified motifs that form binding sites for proteins and other RNAs, and these motifs are often known to be important for the correct functioning of the RNA.[7–16] Therefore, an understanding of small molecule recognition of nonhelical RNA structural motifs will be important for the effective design of inhibitors and modulators of these interactions. In this paper, we report experiments investigating small molecule recognition of RNA tetraloops. We have found that an aminoacridine derivative (AD1) binds to a GAAA tetraloop with 1:1 stoichiometry and micromolar affinity.

RNA tetraloops are four-nucleotide stem loop structures that were first identified from sequence comparisons of ribosomal RNAs.[17,18] More than 55% of loops in the 16S rRNA are tetraloops and more than 50% of these tetraloops have the consensus sequence GNRA.[17] GNRA tetraloops serve as binding sites for proteins and participate in tertiary interactions that contribute to the formation of the proper three-dimensional structures of many RNAs.[19,20] For example, interactions between GNRA tetraloops and RNA structures that are called tetraloop receptors have been observed in structures of the group I intron, the hammerhead ribozyme, and the ribosome.[13–16] These interactions have been demonstrated to contribute to the stability and activity of folded RNAs.[21–24] GNRA tetraloops are targets for proteins, including ricin and the HIV Gag protein, and can contribute indirectly to protein binding by influencing RNA structure.[25–27]

Structures of GNRA tetraloops have been solved by NMR and X-ray crystallography.[13,14,28–34] The sequence and structure of the GAAA tetraloop used in the experiments reported here are shown in Figure 1 and illustrate the general features of tetraloop structures.[35] The first and fourth base form a non-Watson Crick G–A pair, and the second and third bases stack on top of the fourth base. The network of hydrogen bonds and stacking interactions between loop residues causes GAAA tetraloops to be unusually stable.[36]

We investigated the affinity of the small molecules that are shown in Figure 2 for the GAAA tetraloop. We chose three commercially available compounds known to bind RNA: quinacrine and two aminoglycosides, neomycin, and kanamycin. We also chose the acridine

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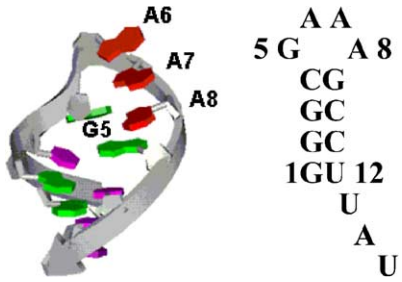

Figure 1. NMR structure of the GAAA tetraloop (PDB ID: 1ZIF)[35] and secondary structure of this tetraloop (right). A8 is substituted by 2-aminopurine in fluorescence experiments that require labeled RNA.

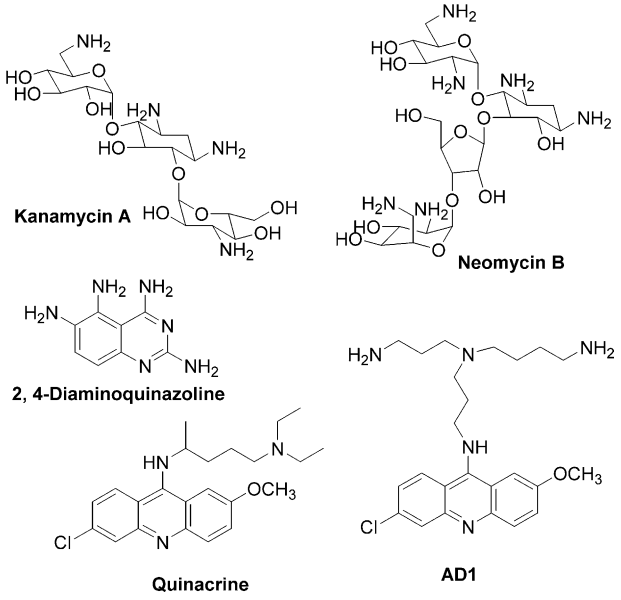

Figure 2. Small molecules used in these investigations.

derivative AD1 and 2,4-diaminoquinazoline because they have been shown to inhibit the HIV-1 Tat–TAR complex by interacting with bulges or loops in TAR RNA.[37,38] AD1 and 2,4-diaminoquinazoline were synthesized following published procedures.[39–41]

The association of each small molecule with the GAAA tetraloop was characterized by fluorescence spectroscopy. Because neither neomycin nor kanamycin is fluorescent, their affinities for the GAAA tetraloop could not be directly measured. There are two general methods to enable fluorescence studies when the small molecule is not fluorescent: a competition experiment can be performed to monitor the displacement of a fluorescent molecule known to bind the target RNA by the nonfluorescent molecule;[42,43] or a direct measurement can be made by monitoring the change in fluorescence of a fluorophore incorporated into the target RNA upon addition of the nonfluorescent molecule.[44,45] Because no GAAA tetraloop ligands were known, we chose to label the GAAA tetraloop by substituting 2-aminopurine for A8. A normal titration was performed in which the fluorescence intensity of 2-aminopurine was monitored as a function of increasing concentrations of neomycin or kanamycin. 2-Aminopurine is sensitive to changes in base stacking interactions, and any change in loop geometry should result in a change in the fluorescence intensity. UV melting experiments were performed to confirm that the 2-aminopurine substitution did not change the stability of the tetraloop (data not shown). We have performed computational docking experiments that identified a binding site for the small molecules in the major groove of the stem region at the base of the loop. These calculations suggest that the 2-aminopurine substitution does not affect the interactions between the small molecule and the GAAA tetraloop because both the amino group and C2 of A8 are in the minor groove. The 2-aminopurine fluorescence signal was quenched by 50% at a neomycin concentration of 30 μM. No change in 2-aminopurine fluorescence was observed upon addition of 1 mM of kanamycin to the GAAA tetraloop.

Association of the fluorescent molecules 2,4-diaminoquinazoline, quinacrine, and AD1 with the GAAA tetraloop was monitored by performing a reverse titration in which the concentration of the small molecule was held constant and the concentration of the GAAA tetraloop was varied. No change in the fluorescence signal of 2,4-diaminoquinazoline was observed upon addition of GAAA tetraloop up to 4 μM. The fluorescence signals of both quinacrine and AD1 were quenched by 50% upon addition of low micromolar concentrations of RNA. Because these initial experiments suggested that AD1 bound with high affinity to tetraloop RNA and quinacrine fluorescence quenching was not consistent at lower quinacrine concentrations, further studies were performed with AD1.

The stoichiometry of the complex formed between AD1 and the GAAA tetraloop was determined by adding increasing concentrations of AD1 to a constant concentration of 2-aminopurine-labeled RNA. For these experiments, the RNA concentration must be significantly above the K_d. Therefore, binding affinity was maximized

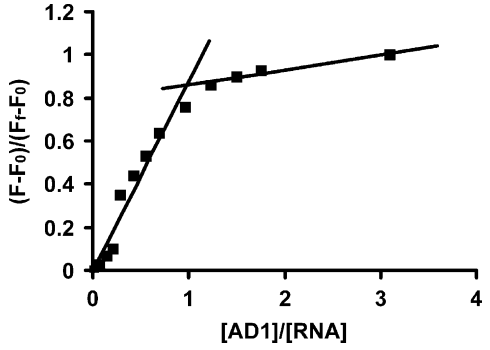

Figure 3. A plot of the fraction of RNA signal quenched versus the molar ratio of AD1 to RNA. This plot was used to determine the stoichiometry of the reaction. F_0 is the initial fluorescence intensity of 2-aminopurine-labeled GAAA tetraloop, F_f is the final fluorescence intensity of the RNA and F is the observed fluorescence intensity. Measurements were performed with an RNA concentration of 20.6 μM in 10 mM Tris·HCl, pH 7.5. The excitation and emission wavelengths were 310 and 370 nm, respectively.

by performing these experiments without added salt (10 mM Tris·HCl, pH 7.5). An example of a stoichiometry experiment is shown in Figure 3. From these experiments the binding stoichiometry was determined to be 1:1.

The equilibrium dissociation constant of the RNA–AD1 complex was measured by performing titrations of AD1 with constant low concentrations of the GAAA tetraloop labeled with 2-aminopurine ($\leqslant 1\,\mu M$). In order to minimize nonspecific electrostatic interactions, binding affinity was measured using higher salt concentrations (10 mM Tris·HCl, pH 7.5, 50 mM NaCl, 5 mM $MgCl_2$) than were used in the stoichiometry experiments described above. The results from one of these experiments are shown in Figure 4. The experimental data were fit with Eq. 1 to determine the dissociation constant.

$$(F - F_0)/(F_f - F_0) = \left((K_d + [R_0] + [S_0]) - \sqrt{(K_d + [R_0] + [S_0])^2 - 4[R_0][S_0]}\right) /2[R_0] \quad (1)$$

F is the fluorescence intensity of the sample, F_0 is the initial fluorescence intensity, F_f is the final fluorescence intensity, K_d is the dissociation constant, $[R_0]$ is the initial RNA concentration, and $[S_0]$ is the total AD1 concentration. A dissociation constant of $1.63(\pm 0.05)\,\mu M$ for the complex formed between the GAAA tetraloop and AD1 was obtained from the average of three titrations.

RNase I and lead acetate footprinting experiments were carried out to investigate the binding site of AD1 on the GAAA tetraloop. RNase I and lead acetate were chosen

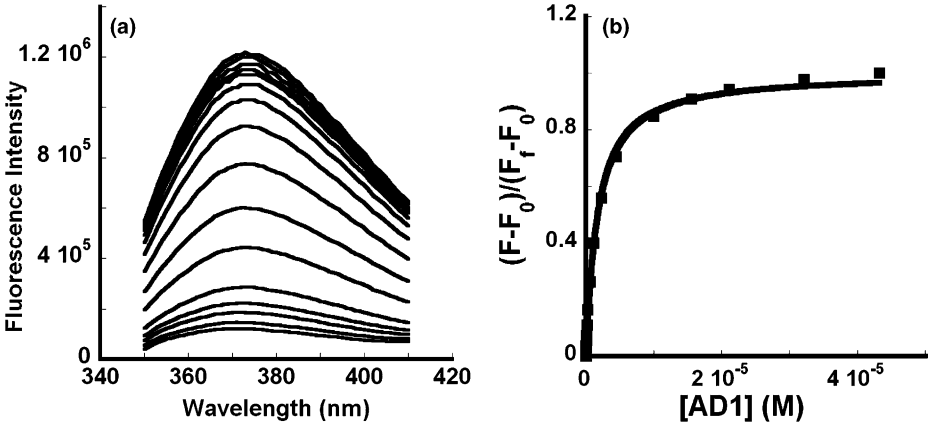

Figure 4. (a) Fluorescence spectra of GAAA tetraloop labeled with 2-aminopurine upon addition of increasing concentrations of AD1. Measurements were performed with an RNA concentration of 0.289 μM and AD1 concentrations ranging from 0 to 43.2 μM in 10 mM Tris·HCl, pH 7.5, 50 mM NaCl, and 5 mM $MgCl_2$ at 20 °C. The intensity of the fluorescence signal of 2-aminopurine decreased upon addition of AD1. (b) Plot of fraction RNA bound versus [AD1]. F_0 is the initial fluorescence intensity of 2-aminopurine-labeled GAAA tetraloop, F_f is the final fluorescence intensity of the RNA and F is the observed fluorescence intensity. The fraction RNA bound was calculated from the fluorescence intensity at 370 nm and the data was fit to Eq. 1 to determine the K_d.

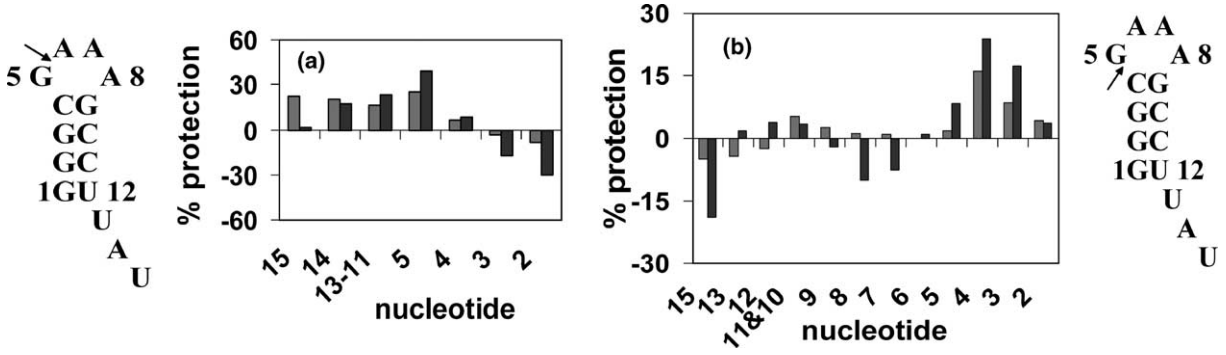

Figure 5. Plots of results of footprinting experiments with (a) RNase I and (b) lead acetate. Reactions were performed with 0.1 μM AD1 (grey bars) and 1 μM AD1 (black bars). Positive numbers indicate reduced RNA cleavage in the reactions containing AD1 compared to the reaction without AD1, while negative numbers indicate enhanced RNA cleavage in the reactions containing AD1 compared to the reaction without AD1. The GAAA tetraloop was 5′-end labeled with ^{32}P. Mixtures of RNA and different concentrations of AD1 were pre-equilibrated for 30 min and then treated at room temperature with 0.01 U RNase I for 10 min or 1 mM lead acetate for 30 min. The reaction mixtures were resolved on a 20% denaturing polyacrylamide gel. Percent protection for each reaction was determined by dividing the difference between the intensity of the RNA band from a reaction containing AD1 and the intensity of the RNA band from the reaction without added AD1 by the intensity of the RNA band from the reaction without AD1.

because they both cleave RNA in single-stranded regions without specificity for any particular base. RNase I cleaves 3′ to all four bases of single-stranded RNA leaving a 5′ hydroxyl group and a 2′,3′-cyclic monophosphate.[46,47] Lead acetate cleaves the phosphodiester bonds of RNA in single-stranded regions, but cleavage may occur in double-stranded regions if they contain weak, bulged, or destabilized base pairs.[48,49] Experimental details are described in the legend of Figure 5. These experiments suggested that binding occurs near the CG base pair closing the tetraloop and the G of the tetraloop. This binding site is similar to one of the binding sites we previously identified for AD1 on stem loop 2 of U1 snRNA[50] and to the binding site of AD1 on the bulge of TAR RNA.[38,39]

These results show that AD1 binds to the CG base pair closing the GAAA tetraloop with low micromolar affinity. The interaction between AD1 and the GAAA tetraloop is probably governed by electrostatic and stacking interactions. Presumably, the flexible amino groups allow favorable interactions with the phosphate groups of the tetraloop, while the orientations of the amino groups in the aminoglycosides kanamycin and neomycin are not appropriate for binding to the GAAA tetraloop. Strengthening either stacking or electrostatic interactions or constraining the geometry of the alkylamino arms of AD1 to match the positions of the phosphate groups in the GAAA tetraloop may result in greater affinity for the GAAA tetraloop. We are currently performing computational docking to suggest modifications of AD1 that will improve affinity for RNA tetraloops and to identify new small molecule scaffolds that bind RNA tetraloops.

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