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Using Selection to Identify and Chemical Microarray to Study the RNA Internal Loops Recognized by 6′-*N*-Acylated Kanamycin A

Matthew D. Disney*[a] and Jessica L. Childs-Disney[a, b]

Herein, we describe our initial steps towards identifying the RNA secondary structure motifs that are recognized by small molecules. We selected members of an RNA 3×3 internal loop motif library that bind kanamycin A, an RNA-binding aminoglycoside antibiotic, by using only one round of selection. A small internal-loop library was chosen because members are likely to be present in other larger, biologically relevant RNAs. We have identified several internal loops of various size and base composition that kanamycin A prefers to bind. The highest affinity structures are two 5′-UU/3′-CU 2×2 internal loops closed by AU pairs. Binding is specific for the selected internal loops with the highest affinities, since binding to the RNA cassette used to display the library or to DNA is >150-fold weaker. Enzymatic mapping experiments also confirm binding of kanamycin A to the internal loops. This method lays the foundation for finding RNA secondary structure elements that bind small molecules and for interrogating factors affecting RNA–ligand interactions. Information from these and subsequent studies will: 1) facilitate the rational and modular design of drugs or probes that bind target RNAs with high affinity, provided the secondary structure of the target is known and 2) give insight into the potential bystander RNAs that aminoglycosides bind.

Introduction

RNA is an important target for developing therapeutics and chemical genetics probes because of the important roles it plays in many biological processes. RNA is the central figure in protein synthesis in which the controlled action of ribosomal, transfer, and messenger RNAs creates all cellular protein. Translation of mRNA into protein is also regulated by RNA. For example, microRNAs regulate RNA lifetime[1,2] by degrading mRNAs through RNA interference.[3,4] Untranslated regions in mRNA can either stimulate or inhibit translation by binding organic ligands,[5–7] proteins, or the ribosome itself.[8] RNA also catalyzes chemical reactions in biological systems.[9,10] In all of these cases, the interplay of RNA secondary and tertiary structure elements is responsible for the RNA's biological activity.[11]

Though most drug discovery efforts are directed towards proteins, RNA is a validated and important drug target that remains largely unexploited. Most small molecules that target RNA selectively bind to noncanonically paired regions. For example, the aminoglycoside antibiotic kanamycin A inhibits protein synthesis by binding to a 1×2 all adenine internal loop and adjacent nucleotides in the A-site of the bacterial ribosome.[12–17] HIV inhibitors bind to a 3-nucleotide bulge or a hairpin in transactivating response (TAR) RNA.[18,19] Viral RNAs from HIV[18–22] and hepatitis C[23,24] as well as catalytic RNAs such as group I introns and RNase P RNAs[25–27] have also been targets for small molecules. In the vast majority of cases, small-molecule RNA inhibitors were identified by screening combinatorial libraries using a traditional medicinal chemistry approach.[19,20,25,26,28–31]

Although these screening efforts have been valuable for finding small molecules that modulate the activity of an RNA, they fail to identify the motifs that small molecules prefer and therefore do not provide enough information to enable the rational design of potent and specific compounds. Therefore, when a modulator of RNA function is desired the target RNA must be subjected to a screening assay to identify lead ligands. These screening assays are often time-consuming and expensive. One potential way to more efficiently design compounds targeting RNA would be to use a database of the RNA motifs that small molecules specifically recognize. The database and the secondary structure of the RNA could be searched in silico to find motifs common to both. Once common motifs are identified between the database and an RNA to which a binder is desired, ligand modules can then be custom-assembled (linked) to bind a series of secondary structure elements in the RNA or used as starting points for focused libraries. Although the structural diversity of RNA makes this an enormous challenge, the idea is intriguing.

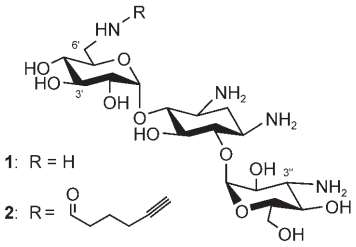
[a] *Prof. Dr. M. D. Disney, Dr. J. L. Childs-Disney*
Department of Chemistry and The New York State Center of Excellence in Bioinformatics and Life Sciences, University at Buffalo
657 Natural Sciences Complex, Buffalo, NY 14260 (USA)
Fax: (+1) 716-645-6963
E-mail: mddisney@buffalo.edu

[b] *Dr. J. L. Childs-Disney*
Current address:
Department of Chemistry and Biochemistry, Canisius College
2001, Main Street, Buffalo, NY 14208 (USA)

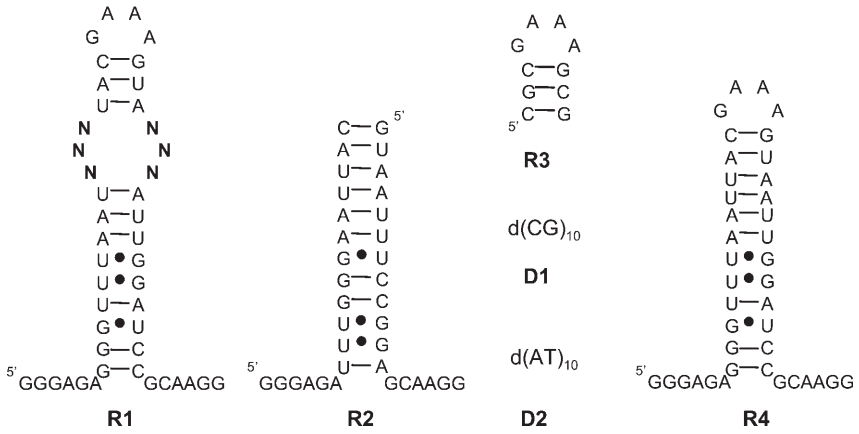
Supporting information for this article is available on the WWW under http://www.chembiochem.org or from the author: details of the chemical synthesis, representative competitive binding assays, and chemical probing of RNA in the presence and absence of **2**.

Modular assembly approaches have been utilized for rational design of small molecules targeting DNA. The Dervan group has used modularly linked polyamides to bind the minor grove of DNA.[32] Their studies were enabled by studies of the natural product distamycin bound to DNA.[33] Identifying small molecule–RNA partners presents a more formidable challenge because of RNA's ability to fold into a diverse set of secondary structures. Therefore, we must be concerned about all secondary structures, not only the canonically paired regions used for DNA–small molecule partners. Fortunately, RNA secondary structures can be rapidly and accurately obtained by prediction through free-energy minimization or phylogenic comparison.[34,35] However, no structural information is presently available to facilitate the identification of small molecule–RNA secondary structure pairs neither is there a database that defines the RNA secondary structure motifs that are targeted by small molecules.

In this report, we describe our initial steps towards constructing a database of RNA–small molecule binding partners to aid the rational design of compounds that target RNA. In order to do so, we have developed a selection-based approach that requires only a single round of selection to identify the RNA secondary structure elements (internal loops, hairpins, and bulges, for example) that bind small molecules. By screening RNA secondary structure libraries, we increase the chances of finding library members in a large biologically active RNA, such as rRNA. This is in contrast to selective exponential enrichment (SELEX) experiments[36,37] that have identified RNAs ranging from 17-mers to 30-mers that bind ligands with high affinity and specificity. Although the SELEX/aptamer approach finds high-affinity RNA–ligand partners, it is unlikely to find these aptamers in a larger, biologically active RNA. In our initial study, we have screened a 4096-member RNA 3×3 internal loop library for binding to kanamycin A that is immobilized through the 6′-amino group onto sepharose resin. We identified an ensemble of structures that binds the aminoglycoside and have scored their relative affinities by using a newly developed microarray-based method. The highest affinity structures have 5′-UU/3′-UC 2×2 internal loops closed by AU pairs.



Scheme 1. Structures of compounds used in this study. **1** is kanamycin A and **2** is 6′-*N*-5-hexynoate kanamycin A that was used in chemical microarray-based assays to study binding to selected RNA internal loops.



Scheme 2. Sequence and structure of oligonucleotides used in this study. The library displaying a random 3×3 internal loop, **R1**; N refers to a random mixture of A, C, G, and U. Chase oligonucleotides, **R2**, **R3**, **R4**, **D1**, and **D2**. Structure **R2** is a duplex that is related to the stems of **R1**; **R3** is a short hairpin with the hairpin sequence and closing base pair in **R1**; **D1** and **D2** are the DNA chase oligonucleotides used. The RNA **R4** is the cassette into which the library was inserted as shown in **R1**.

Results and Discussion

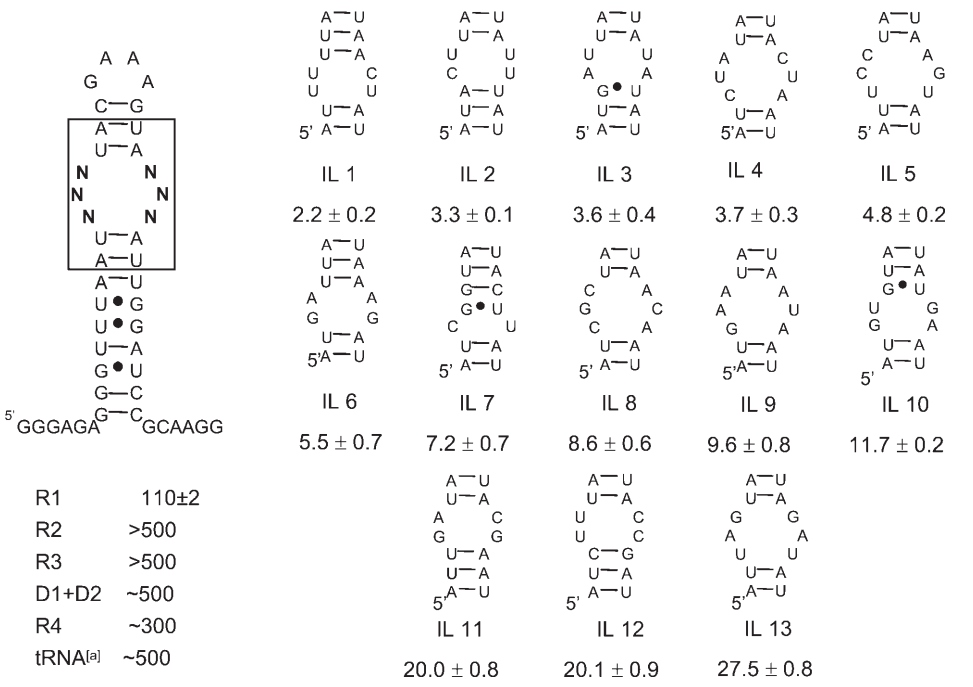
Selection of the RNA internal loops that bind kanamycin A

Selection experiments were completed with kanamycin A (Scheme 1) immobilized onto sepharose resin and an RNA 3×3 internal loop library embedded in a hairpin cassette (**R1**, Scheme 2). This library was designed from a sequence used to identify RNA motifs that have interesting thermodynamic properties by the Bevilacqua group.[38,39] Features of the library include an ultrastable GAAA (GNRA-type) hairpin[40] to facilitate proper folding and single-stranded primer binding sites for amplification through RT-PCR. Kanamycin A was used in our initial study because it is a known RNA-binding ligand and it binds noncanonically paired RNAs like those displayed in the random region of our library. Additionally, this ligand has been immobilized onto resin and used for selection experiments.[37,41]

A large excess of kanamycin A relative to the loading of succinimide esters on the sepharose was used to preferentially immobilize the ligand at the 6′-NH_2 position, which is the most nucleophilic. It should be noted that when kanamycin A is acetylated at the 6′ position, binding to an oligonucleotide mimic of the bacterial A-site is reduced several orders of magnitude relative to the parent aminoglycoside.[42] The reduction in binding affinity is due to the elimination of important contacts that the 6′-amino group forms with the A-site.[13,16,17] Therefore, we have challenged our system to find 6′-acetylated kanamycin A–RNA secondary structure partners by using the modified aminoglycoside as bait to pull down members of the 3×3 internal

loop library. The 6′-NH_2 group has the potential to be used in future experiments as a reactive group for conjugation to link small molecule modules together to target a larger RNA.

Selections were completed by incubating a solution that contained 5′-end ^{32}P-labeled **R1** in buffer with resin-immobilized kanamycin A. In addition to **R1**, a variety of other oligonucleotides were added to the incubation solution (**R2**–**R4, D1, D2**). We found that on average only 150 members (~4%) of the 4096-member 3×3 internal loop library bound to immobilized kanamycin A based on the percentage of the initial radioactivity that eluted with kanamycin A. This suggested that we did not have to increase the stringency of our selection by adding higher concentrations of chase oligonucleotides **R2**–**R4**, **D1**, and **D2** and that the random regions in the library are being recognized. The small number might be due to the manner in which the ligand was immobilized onto the resin (impairs the 6′-amino group from forming stabilizing contacts with RNA).

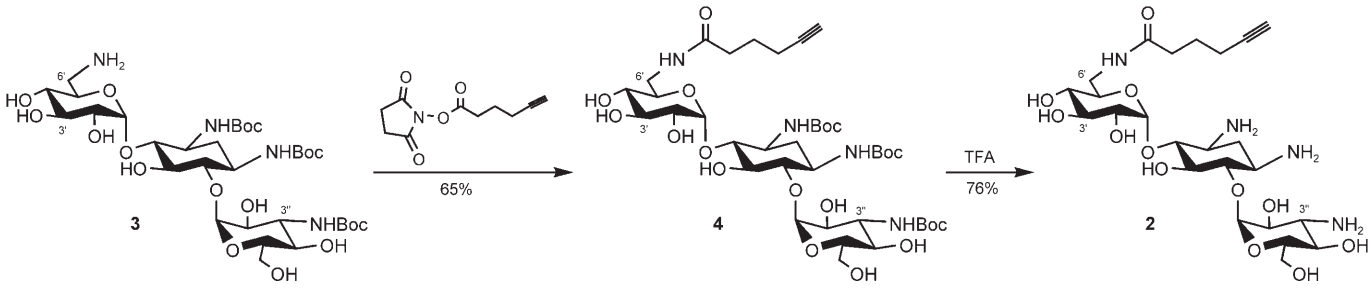


Scheme 3. Secondary structures of the RNA internal loops and the closing base pairs that were selected to bind immobilized kanamycin A. Secondary structures were modeled by using free-energy minimization with the program *RNAStructure*. The internal loop library is shown on the left; boxed nucleotides are shown for the selected internal loops that bind to kanamycin A (right). Values under each loop represent the IC_{50} (nM). [a] The tRNA is bulk tRNA from yeast.

Selected RNAs were amplified through RT-PCR, cloned into a vector, and sequenced. The secondary structures of the selected RNAs were predicted by the RNA secondary structure prediction program *RNAstructure*.[34] A variety of structures were identified including a 1×1 CU internal loop, seven 2×2 loops with **IL1** and **IL2** having the same loop sequence but different orientation of a closing base pair, and five 3×3 loops (Scheme 3).

Studying the binding of selected RNA internal loops to kanamycin A

To score the binding affinity of each RNA internal loop, we developed an array-based method to quickly determine relative affinities. Microarrays have been used in a variety of experiments to probe the affinities of protein and RNA to small molecules in a rapid and highly parallel manner requiring minimal amounts of the analyte and ligand.[43–47] To complete microarray-based affinity experiments with kanamycin A we required a kanamcyin A derivative that could be selectively immobilized onto an array surface. We envisioned that 6′-*N*-5-hexynoate kanamycin A (**2**, Scheme 1) would allow for defined immobilization through reaction of the alkyne handle with azide-displaying glass slides using a Huisgen dipolar cycloaddition reaction, or "click chemistry".[48] The synthesis of **2** was completed as illustrated in Scheme 4. We used 1,3,3′′-tri-*N*-(*tert*-butyl dicarbonyl) kanamycin A (**3**)[49] as starting material in the synthesis since all amino groups except for the one at the 6′ position are protected as acid-labile Boc groups. Compound **3** was treated with *N*-succinimidyl-5-hexynoate to afford **4** in 65% yield. This compound was deprotected with trifluoracetic acid in 76% yield to afford the final product **2**. A neamine derivative was



Scheme 4. Synthesis of 6′-*N*-5-hexynoate kanamycin A (**2**) used for immobilization onto azide-displaying glass slides by click chemistry. Boc = *tert*-butoxycarbonyl ; TFA = trifluoroacetic acid.

also synthesized using the same synthetic sequence and was subsequently labeled with fluorescein by reaction with fluorescein isothiocyanate. (See the Supporting Information for all small-molecule syntheses and characterization.)

Fluorescein-labeled 6′-*N*-hexynoate neamine (neamine-Fl) was synthesized to test click-chemistry immobilization conditions because the fluorescent signal allowed a direct readout of surface loading (Figure 1). A solution of neamine-Fl was spotted onto azide-displaying agarose slides and reactions were completed in the presence or absence of each reagent required for reaction. Loading of the labeled neamine-Fl onto the slide surface was only observed when all of the click chemistry reagents were added (sodium ascorbate, $CuSO_4$, and the tris((1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl)amine (TBTA) ligand). Signals that approach background were observed when any one of the reagents was removed from the spotting solution. These results indicate that 6′ hexynoate neamine-Fl is reacting with the surface through triazole formation. We observe similar results when 6′-*N*-5-hexynoate kanamycin A is immobilized and then probed for binding to a radioactively labeled selected RNA.

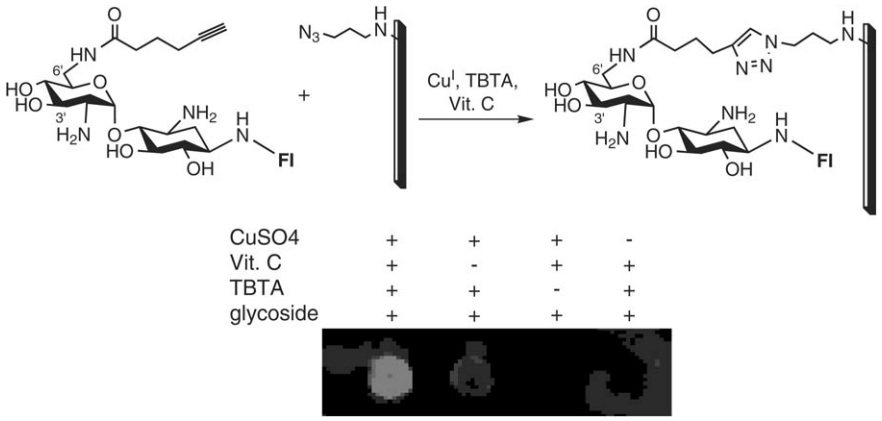


Figure 1. Optimization of click chemistry conditions for immobilization of fluorescein-labeled 6′-*N*-5-hexynoate neamine onto azide-displaying surfaces. Top, the reaction of FITC-labeled 6′-*N*-5-hexynoate neamine with azide-displaying agarose slides under click chemistry conditions. (The reaction was not analyzed for its isomeric content and therefore a single FITC isomer is drawn for simplicity.) Bottom, an image of an array after reaction of FITC-labeled 6′-*N*-5-hexynoate neamine with azide-displaying agarose slides with a variety of conditions. The concentrations of each component in these reactions are 5 mM sodium ascorbate, 1 mM $CuSO_4$, 100 μM TBTA, and 80 μM of 6′-*N*-5-hexynoate neamine-Fl.

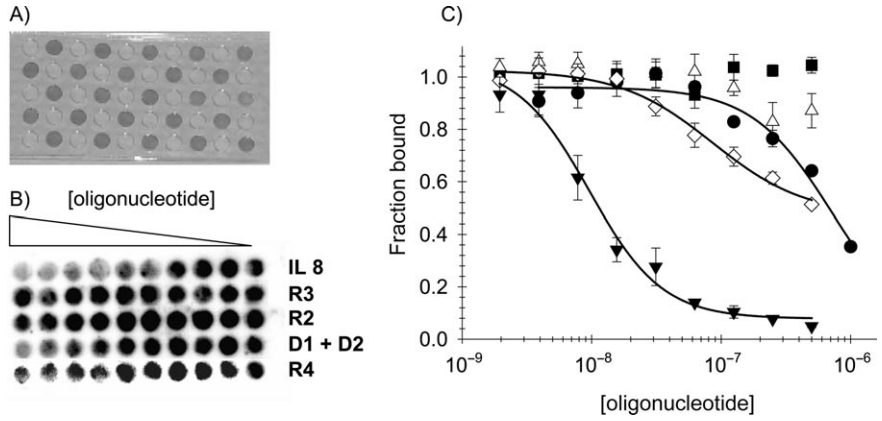


Figure 2. A) A photograph of a silicon gasket affixed to a microarray with half of the wells filled with a solution of dye. B) An autoradioagram of an array-based competitive binding assay completed with five different oligonucleotides. C) The fit of the data for IL 8 and the control oligonucleotides including the cassette. ■: **R3**, △: **R2**, ●: **D1** + **D2**, ◇: **R4**, ▲: **IL 8**. Errors are standard deviations of three independent experiments and in some cases are smaller than the data points.

Typically microarray experiments can be used to probe the binding of many small molecules to a single or few analytes at once. In order to increase the number of conditions that can be probed on a single array, we affixed a silicon gasket that created 50 microwells on the surface of each chip (Figure 2). These microwells converted the array into a miniaturized 50-well plate that is easily functionalized to display **2** by the cycloaddition reaction. Compound **2** was immobilized in each microwell at a constant concentration. Binding experiments were completed by delivering 10 μL of a solution containing serially diluted concentrations of the internal loop of interest and trace 5′-end ^{32}P-labeled **IL 7** into each microwell. Dose response curves were constructed and fit to determine the concentration of competitor that inhibited 50% of the binding of the radioactively labeled RNA (Scheme 3, Figure 2, and the Supporting Information). Figure 2B is a representative autoradiogram for binding selected RNAs relative to binding of control oligonucleotides **R2**, **R3**, **D1** and **D2**, and **R4**, the cassette without an internal loop. All selected loops have at least a >20-fold lower IC_{50} than tRNA or any of the control oligonucleotides used (Scheme 3). tRNA has approximately twice the number of nucleotides as our loop library, and its weak affinity for kanamycin A suggests that binding to the loops is not due to forming nonspecific electrostatic contacts to the phosphodiester backbone. Moreover, each of the selected RNAs binds more tightly to kanamycin A than the entire library (**R1**) which has an IC_{50} of 110 nM. These results suggest that our method selects for specific RNA internal loop–ligand interactions and eliminates the selection of weaker binders.

The two highest affinity sequences, **IL 1** and **IL 2**, have IC_{50} values of 2.2 and 3.3 nM, respectively. Both are 5′-UU/3′-UC 2× 2 loops but they differ in the orientation of one of the closing

AU pairs. It is interesting to note that for tandem GA pairs the orientation of the closing base pairs dramatically affects the structure of the internal loops formed.[50,51] It appears that this is not the case when a single closing pair in **IL1** and **IL2** is changed based on this data. The five highest affinity binders (**IL1**–**5**) contain two U's in the same position on opposite sides of the loop (Scheme 3). **IL13** also displays this sequence pattern; however, the U's are flanked by purines. It is possible that their presence affects the loop structure or dynamics and is the cause of decreased affinity. If one of the U's on opposite sides of the loop in **IL1** and **IL2** is replaced with a C and the closing base pair is changed from AU to GC, the resulting loop, **IL12**, binds approximately ten times more weakly. This suggests that the aminoglycoside is forming contacts with loop nucleotides and/or the closing base pair or that the structure of the loop is altered when the nucleotides are mutated.

The binding site of **2** was determined by enzymatic probing of **IL7** and **R4** in the presence or absence of **2** with RNase A and S1 nuclease. Results show that only nucleotides in the loop and the surrounding base pairs in **IL7** are protected from cleavage. No nucleotides in the cassette (**R4**) are protected (Figure 3). These results coupled with the relative binding measurements further suggest that we have selected small-molecule partners for a specific RNA internal loop. We have also observed decreased modification by diethyl pyrocarbonate (DEPC) of loop nucleotides in **IL1** and **IL11** when **2** is present. DEPC modifies U's at slightly basic pH.[52,53]

A variety of biologically important RNAs bind aminoglycosides including the A-site in bacterial ribosomes,[12,15] and TAR[18] and regulator of virion responsive element[54] RNAs from HIV. Aptamers to tobramycin and kanamycin have also been selected.[37,41] Interestingly, the aptamers for kanamycin and tobramycin,[37,41] TAR RNA,[18] and the A-site[12,13,16] all have unpaired U's in the sites that bind to the aminoglycoside. The consensus region in one tobramycin aptamer contains a ten-nucleotide hairpin in which four of the hairpin nucleotides are U's.[37] In TAR RNA, two of the three nucleotides in the bulge that binds tobramycin are U's, and there is a 1×1 UU internal loop in the A-site in bacterial ribosomes. Structural studies of aminoglycosides bound to the A-site show that the aminoglycoside forms direct contacts to the UU pair (U1406–U1495) through the 2-deoxystreptamine ring common to all aminoglycosides.[16] Based on these studies it is clear that the aminoglycosides bind to a wide array of noncanonically paired RNA structures, with a preference for U. This might be due to the conformational flexibility of aminoglycosides, allowing them to fit into a variety of RNA folds.[22] Taken together with our study, these collective data suggest that aminoglycosides prefer RNAs that have noncanonically paired or unpaired U's. We have identified a consensus 2×2 RNA internal loop sequence displaying unpaired or noncanonically paired U's that binds 6′-*N*-acetylated kanamycin A. These results further our understanding of the RNA internal loops bound by ligands and might also give insight into off-target effects when aminoglycosides bind to bystander RNAs. This information could aid the design of less toxic aminoglycosides.

Implications

Herein, we have described the development of methods to identify RNA–small molecule partners and to quickly score their relative affinities. Results from this study represent our first entry into constructing a database of small molecule–RNA secondary structure motif interactions. This rational approach has the potential to allow more efficient identification of small molecule lead compounds that modulate RNA structure and function.

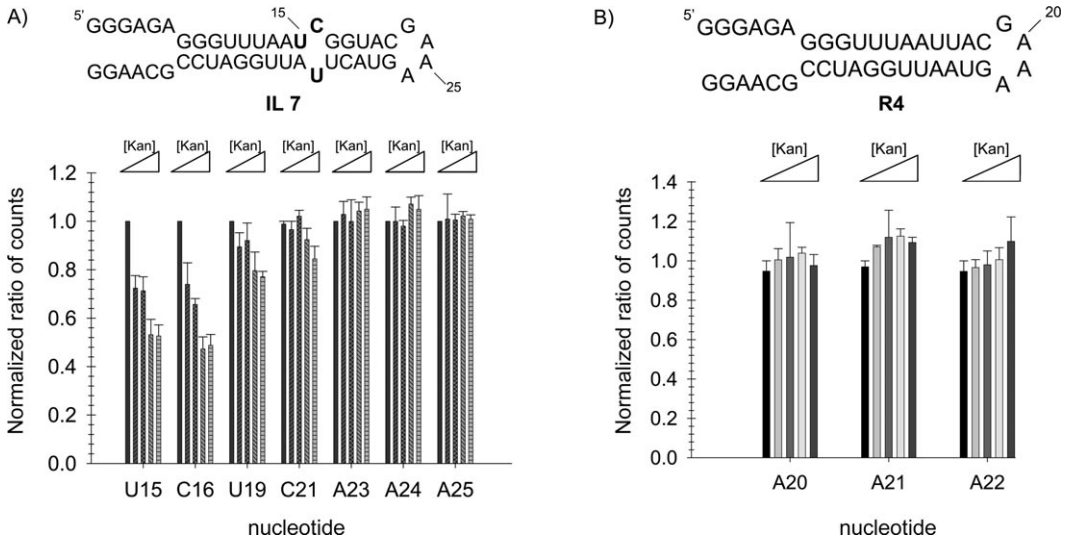


Figure 3. Nuclease mapping results in the presence and absence of 6′-hexynoate kanamycin A. Bold nucleotides are protected from modification in the presence of compound. Results show that binding with **IL7** occurs only in the randomized regions from the original library (A). No nucleotides are protected from modification in the hairpin cassette (**R4**), B, as expected. Plots are ratio of counts for each nucleotide as a function of 6′-*N*-5-hexynoate kanamycin A. The concentrations of compound are 0, 0.1, 1, 10, and 100 μM.

Experimental Section

Selection and nucleic acid synthesis

General methods I: Nanopure water was used in all experiments. All solutions for buffers were prepared with DEPC-treated water to insure the absence of nucleases. All DNA oligonucleotides were purchased from Integrated DNA Technologies (IDT, Coralville, IA) and were purified by denaturing 17% PAGE. DNAs were visualized by UV shadowing, and the products were extracted from the gel slice by tumbling overnight in sterile NaCl (0.3 M) at 4 °C. tRNA XSA from Baker's yeast used in the microarray binding experiments was purchased from Sigma–Aldrich. *N*-hydroxyl succinimde-activated sepharose was purchased from Amersham. Reverse transcriptase was from Life Sciences, Inc. (St. Petersburg, FL). Oligonucleotide extinction coefficients were determined on the HyTher server available from the SantaLucia laboratory (HyTher version 1.0, Nicolas Peyret and John SantaLucia, Jr., Wayne State University).[55,56] Parameters therein are based on extinction coefficients of nearest neighbors in RNA.[57]

General methods II: See the Supporting Information.

RNA oligonucleotide synthesis: The oligonucleotides **R1** and **R4** and the two oligonucleotides that comprise the duplex **R2** were synthesized by run-off transcription of synthetic DNA templates using T7 RNA polymerase.[58] Oligonucleotide **R3** was purchased from IDT. Purity of all oligonucleotides was determined to be >90% from analytical kinase experiments completed as previously described.[59] Plasmids encoding the selected sequences were amplified through PCR with a primer that contained a recognition site for T7 RNA polymerase. (See "Selection of RNA Internal Loops" for complete details.) The products of the PCR reaction were then used directly for transcription using the same methods described above. Reactions were purified on a denaturing 17% polyacrylamide gel. Products were visualized and extracted from the gel as described in the General Methods Section.

Attachment of aminoglycosides to N*-hydroxyl succinimde-activated sepharose*: A suspension (2.4 mL) of resin in isopropanol (1.2 mL of resin, loading 18 μmol mL^{-1}) was placed into a BioRad polyprep chromatography column and washed with H_2O (2×10 mL). To the washed resin was added kanamycin A (50 mg, 105 μmol) in Na_2CO_3 buffer (5 mL, pH 9.0), and the resin was tumbled overnight at 4 °C. After incubation, the resin was washed with buffer (2×10 mL), and the remaining succinimide esters were quenched with ethanolamine (5 mL, 10 mM) in 5% Na_2CO_3 (1 h at room temperature with tumbling). The resin was washed with water and then sequentially washed with NH_4OAc (10 mL, 10 mM, pH 4.5) and Tris·HCl (10 mL, 10 mM, pH 8.0). These steps were repeated three times, and the resin was stored in Tris·HCl (10 mM, pH 8.0) at 4 °C. Excess aminoglycoside relative to succinimide esters loaded on the resin and incubation at 4 °C were used for conjugation to allow reaction to occur preferentially at the 6′-amino position of kanamycin A. Previous experiments have shown that this position is the most reactive when aminoglycosides are conjugated to dyes that contain succinimide esters and to resin.[37,45,60]

Selection of RNA internal loops that recognize kanamycin A: Each of the oligonucleotides was refolded separately in hybridization buffer (HB) containing Na_2HPO_3 (8 mM, pH 7.0), EDTA (1 mM), and NaCl (185 mM) by heating for 5 min at 65 °C and then cooling to room temperature on the benchtop. Oligonucleotides were then transferred to 4 °C and mixed together. Before the oligonucleotides were hybridized with the resin, BSA (200 μg) was added to the samples. Each selection experiment was completed with 10 pmol of 5′-end labeled **R1** and 20-fold higher concentration of each control oligonucleotide (**R2**–**R4, D1, D2**) over the concentration of **R1**.

A 100 μL aliquot of resin slurry (10 μL of resin) was placed into a 2.5 mL BioRad column and washed three times with HB (1 mL, cooled to 4 °C). The resin was then washed as follows: 1) 2.5 mL of HB containing 10 pmol of **R1**; 2) 5 mL of HB; 3) 2.5 mL of HB containing 200 pmol of all RNA chase oligonucleotides (**R2**–**R4**); 4) 2.5 mL of HB containing 200 pmol of all DNA chase oligonucleotides (**D1**, **D2**); 5) 2×5 mL of HB. At this stage, liquid scintillation counting was used to ensure that only background radioactivity was being eluted from the column. Bound RNAs were eluted by delivering a 2 mL solution of HB containing 1 mM of kanamycin A. Scintillation counting of the elution showed that only a small percentage of **R1** (~4%) bound to the immobilized ligand.

The eluate was concentrated to 500 μL with 2-butanol and ethanol precipitated with glycogen (100 μg) as a carrier. The sample was resuspended in H_2O (100 μL) and treated with RQ DNase I (RNase-free, Promega; 1 unit) by incubation at 37 °C for 3 h. An equal volume of stop buffer (89 mM Tris borate, 1 mM EDTA, 7 M urea) was added to the reaction and the sample incubated at 65 °C for 10 min to inactivate the DNase. After phenol/chloroform extraction and ethanol precipitation, the sample was used as a template in RT-PCR reactions.

RT-PCR reactions were completed as described previously.[38,39,61] Control experiments were run to ensure that there was no contamination in the samples, including no template and template without RT controls. Primers used for RT-PCR experiments were: PCR primer, 5′-GGCCGAATTCTAATACGACTCACTATAGGGAGAGGGTTTAAT, containing a T7 promoter for transcription with RNA polymerase; RT primer, 5′ CCTTGCTCCAAT. RT reactions were completed by annealing the isolated RNA solution (20 μL) and a solution of RT primer (2 μL, 100 μM) at 70 °C for 10 min followed by incubation on ice for 10 min. Then dNTPs (1.6 μL, 25 mM), BSA (0.8 μL, 10 mg mL^{-1}), 10x RT buffer (4 μL, supplied by the manufacturer), and RT or H_2O for no RT controls (0.1 μL) were added. The reaction was allowed to proceed for 1 h at 60 °C at which point the reaction was quenched by heating at 95 °C for 3 min. PCR amplification was completed by adding PCR primer (4 μL, 100 μM), RT primer (2 μL, 100 μM), $MgCl_2$ (0.6 μL, 250 mM), Taq polymerase (0.2 μL), H_2O, (13 μL), and 10x PCR buffer (6 μL). Twenty cycles of PCR were completed at 95 °C for 1 min/72 °C for 1 min. Reactions were analyzed by running an aliquot on a denaturing 17% polyacrylamide gel stained with SYBR gold or ethidium bromide. Only experiments in which the negative controls contained no product were carried on towards cloning.

RT-PCR products were then digested with *Bam*HI and *Eco*RI and were ligated into a pUC19 vector that was digested with the same enzymes.[38,39,61] The plasmids were transformed into DH5α competent *E. coli* and plated on Luria Bertani (LB) plates containing ampicillin, X-gal, and IPTG. White colonies were used to inoculate LB cultures (2.5 mL) containing ampicillin and were grown overnight at 37 °C. Plasmids were isolated from bacteria using an Eppendorf fast plasmid mini kit. Sequencing reactions were completed by the Roswell Park Cancer Institute's Biopolymer lab.

Microarray-based experiments

Preparation of azide-functionalized glass slides: Aminosilane slides (Sigma) were coated with melted agarose solution (1%, ca. 2 mL).[62] Slides were placed on the bench and allowed to dry to a clear film. The agarose-coated slides were submerged in an aqueous $NaIO_4$ solution (20 mM) for 30 min[62] followed by extensive washing in water (3×30 min with frequent water changes). Slides

were then submerged in 10% aqueous ethylene glycol for 1.5 h and were washed with water as described above. To display azides, slides were reacted with 3-azidopropylamine (20 mM) in $NaHCO_3$ (0.1 M) overnight. The following morning, slides were quenched by incubation for 3 min in $NaCNBH_3$ solution (100 mg in 10 mL ethanol plus 40 mL phosphate-buffered saline). Slides were washed with water and left to dry to a clear film on the benchtop.

Click reactions on the slide surface: Click reaction conditions on the slide surface were optimized using a fluorescein isothiocyanate (FITC)-neamine substrate by varying the concentrations of $CuSO_4$, TBTA, and sodium ascorbate or tris(2-carboxyethyl)phosphine in phosphate buffer (10 mM) containing 10% glycerol. Solutions were incubated on the slide surface for 2 h at room temperature, and then washed with SDS (0.2 M) for 15 min and with water for 15 min. The slides were dried in a stream of air and imaged using a Kodak Gel Imaging System equipped with a 535 nm filter. The optimal reaction conditions found were 1 mM $CuSO_4$, 100 μM TBTA, and 5 mM sodium ascorbate. Reactions are specific because no reaction occurs when Cu^{2+} or TBTA are removed from the reaction, and only a slight signal is observed in the absence of sodium ascorbate (Figure 1). These conditions were used to immobilize **2**.

Determination of IC_{50}'s and relative binding affinities: In order to determine which concentration of **2** was optimal for competitive binding assays, serially diluted **2** was applied to the chip surface and allowed to react for 2 h at room temperature inside the microwell of a silicon gasket (GRACE Biolabs, CWCS 50R-1.0, C-24780; Figure 2, Supporting Information). Each of the wells was washed twice with water and the slide was dried in air.

The 5′-end ^{32}P-radiolabeled **IL7** was annealed in 1X HB containing BSA (40 μg mL^{-1}) at 60 °C for 5 min and allowed to slowly cool on the bench top for 10 min. It is important to note that the hybridization buffer contains 1 mM EDTA, or 50 equiv compared to the amount of Cu^{2+} used for the click reaction. EDTA was included to ensure that Cu^{2+} does not bind to the aminoglycoside and cleave the RNA phosphodiester backbone.[63] The annealed RNA oligonucleotide was hybridized with the chip surface for 30 min at room temperature. Slides were washed twice for 2 min with 1X HB containing BSA (40 μg mL^{-1}). Slides were dried in a stream of air and imaged using a BioRad FX Imager. This direct binding measurement showed that delivering 400 nL of a 2.5 mM solution of **2** to the array was sufficient to observe binding to radiolabeled **IL7** (Supporting Information).

IC_{50}'s were determined by incubating serially diluted competitor RNA and trace radiolabeled **IL7** in microwells formed using a silicon gasket. Briefly, the internal loop for which the IC_{50} was determined was annealed in 1X HB containing BSA (40 μg mL^{-1}) as described above. **IL7**, which was used to optimize the concentration of **2** in the spotting solution, was annealed separately in the same manner. After slow cooling on the benchtop for 10 min, the radiolabeled internal loop was added to serially diluted competitor RNA and the sample applied to a microwell. The solution was allowed to equilibrate with the surface for 30 min at room temperature after which the slide was washed twice with 1X HB BSA (40 μg mL^{-1}). After drying in a stream of air, the slides were exposed to a phosphorimager plate and imaged using a Bio-Rad FX Imager. The data were quantified by using Bio-Rad's QuantityOne software, and the IC_{50} values were determined from SigmaPlot's four-parameter logistical curve fit.

Enzymatic probing of RNA

Hydrolysis and nuclease T1 ladders: Hydrolysis ladders were generated by incubating 5′-end ^{32}P-radiolabeled RNA oligonucleotides in $NaHCO_3$ (150 mM, pH 10) and EDTA (1 mM) for 4 min at 95 °C. Nuclease T1 cleaves after single stranded guanosine nucleotides. A T1 ladder was generated under denaturing conditions by incubating radiolabeled RNA oligonucleotides in sodium citrate (20 mM, pH 5), urea (7 M), and EDTA (1 mM), with T1 (0.25 u) at 55 °C for 5 min. Both reactions were quenched by adding equal volumes of stop buffer.

RNase A and S1 nuclease mapping: The binding site(s) of **2** was determined by using a nuclease protection assay. For RNase A mapping experiments, radiolabeled oligonucleotide was annealed as described above. After slow cooling, serially diluted concentrations of **2** were added and the resulting solution allowed to equilibrate for 30 min at room temperature. Approximately 6 μunits of RNase A were added and the reaction was allowed to proceed for 10 min. The reaction was quenched by addition of an equal volume of stop buffer and the products separated on a denaturing 17% polyacrylamide gel.

For S1 nuclease mapping experiments, the radiolabeled oligonucleotide was annealed as described, except the buffer used was sodium acetate (50 mM, pH 4.5), NaCl (280 mM), and $ZnSO_4$ (4.5 mM). The solution was allowed to equilibrate for 30 min at room temperature after the addition of serially diluted concentrations of **2** and then 10 units of S1 were added. The reaction was quenched after 10 min at room temperature, and the products were separated on a denaturing 17% polyacrylamide gel.

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