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Defining RNA motif–aminoglycoside interactions via two-dimensional combinatorial screening and structure–activity relationships through sequencing

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Abstract

RNA is an extremely important target for the development of chemical probes of function or small molecule therapeutics. Aminoglycosides are the most well studied class of small molecules to target RNA. However, the RNA motifs outside of the bacterial rRNA A-site that are likely to be bound by these compounds in biological systems is largely unknown. If such information were known, it could allow for aminoglycosides to be exploited to target other RNAs and, in addition, could provide invaluable insights into potential bystander targets of these clinically used drugs. We utilized two-dimensional combinatorial screening (2DCS), a library-versus-library screening approach, to select the motifs displayed in a 3×3 nucleotide internal loop library and in a 6-nucleotide hairpin library that bind with high affinity and selectivity to six aminoglycoside derivatives. The selected RNA motifs were then analyzed using structure–activity relationships through sequencing (StARTS), a statistical approach that defines the privileged RNA motif space that binds a small molecule. StARTS allowed for the facile annotation of the selected RNA motif–aminoglycoside interactions in terms of affinity and selectivity. The interactions selected by 2DCS generally have nanomolar affinities, which is higher affinity than the binding of aminoglycosides to a mimic of their therapeutic target, the bacterial rRNA A-site.

Keywords

Nucleic acids; RNA; Aminoglycosides; Drug design; Antibacterials; High throughput screening

1. Introduction

Potential RNA targets are present in the transcriptomes of many organisms; however, these targets are rarely exploited with small molecules to modulate function.^{1,2} The bacterial ribosome is the most thoroughly studied RNA target as its function is modulated by various classes of small molecules.³ In particular, aminoglycoside antibiotics bind the ribosomal A-site, affecting A-site dynamics and proof reading of the codon–anti-codon interaction.^{4–9} One outstanding challenge in chemical biology is the development of compounds that modulate the function of non-ribosomal RNAs. In order to develop such compounds, however, more information on the types of small molecules that bind RNA is required.

Moreover, the complementary data are also needed: the types of RNA motifs that bind small molecules and are likely to be found in a transcriptome.

Previously, our research group developed an approach termed two-dimensional combinatorial screening (2DCS) that probes small molecule and RNA motif spaces simultaneously such that privileged small molecule space and privileged RNA motifs are identified.^{10–12} The RNA motif–small molecule interactions identified from our 2DCS studies have facilitated the design of bioactive small molecules that target RNAs that cause myotonic dystrophy type 1 (DM1), fragile X-associated tremor ataxia syndrome (FXTAS), and Huntington's disease (HD).^{13–16}

In order to further expand our understanding of RNA–small molecule interactions, we completed 2DCS studies on six aminoglycoside derivatives (Fig. 1). The compounds were probed for binding to RNA motifs that are derived from 3×3 nucleotide internal loop and 6-nucleotide hairpin libraries (Fig. 2). These studies identified high affinity, selective RNA motif–aminoglycoside interactions that were annotated by using structure–activity relationships through sequencing (StARTS) and experimentally determined binding affinities.^{12,17,18} In general, selected aminoglycoside–RNA motif interactions are of comparable or higher affinity than the affinities of aminoglycosides for their therapeutic target, the bacterial rRNA A-site. Furthermore, the hairpins and internal loops that were selected to bind to each aminoglycoside have similar affinities, suggesting that aminoglycosides do not have a large bias for one over the other.

2. Results and discussion

In order to complete 2DCS studies, small molecules that can be site-specifically immobilized onto microarray surfaces are required. Previously, we have shown that a Huisgen dipolar cycloaddition reaction (Cu(I)-catalyzed click chemistry)^{19,20} can be used to anchor azide-functionalized small molecules onto alkyne-functionalized surfaces.^{10,12} Thus, a small library of aminoglycosides (AmG), each appended with an azido group was employed. The library was previously synthesized²¹ and contains two derivatives of paromomycin with either an azido group at the 6 or the 5 positions (PAR I and PAR II, respectively), 6 azido-amikacin (AMI), 5 azido-ribostamycin (RIB), 6-azido-apramycin (APR), and 3-azido-streptomycin (STR) (Fig. 1). Each compound is derived from an antibiotic that targets the bacterial ribosome.^{4–9} Amikacin, ribostamycin, apramycin, and paromomycin bind the bacterial A-site while streptomycin binds the 16S rRNA and interferes with binding of formyl methionine tRNA.^{22,23}

Small molecule microarrays were constructed by delivering five different concentrations of each compound to the slide surface (Figs. 1B and 3A). The arrays were then probed ³²P-labeled 3×3 nucleotide internal loop (**1**) and the 6-nucleotide hairpin (**2**) libraries simultaneously in the presence of unlabeled competitor oligonucleotides (**3–8**) (Fig. 2). Competitor oligonucleotides ensure that selected interactions occur between the small molecule and the randomized regions in the RNA motif libraries (N's; Fig. 2) and not to regions common to all library members. Competitors are present in threefold excess over the amount of small molecule delivered to the array surface and in >1000-fold excess over the moles of **1** and **2**. Because each compound was spotted at five different concentrations and probed for binding to **1** and **2**, over 245,000 interactions were probed simultaneously on a single microarray surface (6 compounds $\times$ 5 concentrations $\times$ 2 RNA libraries $\times$ 4096 RNA motifs in each library) (Fig. 3A).

Each aminoglycoside binds members of the RNA motif libraries under our highly stringent conditions (Fig. 3A). The relative signals on the array correlate with the number of ionizable

amino groups displayed by the compounds: PAR I $\approx$ PAR II $>$ RIB $\approx$ APR $>$ STR $\approx$ AMI (approximate at all small molecule loadings) (Fig. 3B). Large signals indicate either selection of very high affinity interactions or that the compound binds a large number of RNA motifs.

The lowest ligand loading at which signal can be observed above background is 100 pmol. Previous studies have shown that the interactions selected at the lowest ligand loading are the highest affinity.¹⁰ Therefore, the RNA motifs bound at 100 pmole loading were excised from the microarray surface, cloned, and sequenced (Fig. 3A). Excised RNAs were easily amplified by RT-PCR, and RT-PCR product is only observed in the presence of reverse transcriptase, indicating that the amplified products are exclusively derived from the input RNA (Fig. 3C).

At least 40 selected RNA motifs were sequenced for each aminoglycoside and include members from both RNA libraries (**1** and **2**, Fig. 2) (Tables S1–S12). Analysis of the secondary structures from these selections showed that there is no preference for 1×1 , 2×2 , or 3×3 nucleotide internal loops (derived from **1**) or for a 4- or 6-nucleotide hairpin loops (derived from **2**). The lack of bias for a particular secondary structure has been observed previously for aminoglycosides.^{24–30}

The finer details of the features that constitute privileged RNA motif space for each aminoglycoside were determined by StARTS as calculated by the RNA-PSP program.^{17,30} StARTS analyzes features in the selected RNA motifs that bind to a small molecule by comparing the rate of occurrence of a feature of interest in selected sequences to the rate of occurrence in the starting library.^{17,18} Each feature is assigned a *Z*-score, which is a measure of statistical significance. Each individual RNA motif has multiple statistically significant features, and the *Z*-scores of each feature are summed to afford a Sum *Z*-score. The Sum *Z*-scores are then normalized to the highest value to afford a Fitness Score for each RNA motif–small molecule interaction (Fig. 4). Fitness Score plots for each compound show that the selected interactions have high Fitness Scores as expected (minimally the top 25% of all possible interactions; Fig. 4).

The most significant features in the RNAs selected for **1** and **2** are summarized in Tables S13 and S14, respectively. (All trends with two-tailed *p*-values < 0.00003 are listed in Tables S15–S26.) Interestingly, there are more trends in the internal loops selected to bind PAR I and PAR II than there are for hairpins. The opposite trend is observed for the remaining aminoglycosides. Based on StARTS analysis, APR can accommodate binding to more diverse hairpin motif space than other aminoglycosides (46 trends with the highest statistical significance) while PAR II can accommodate binding to more diverse internal loop motif space (20 trends with the highest statistical significance).

The affinities of the 10 RNAs with the highest Fitness Scores for each aminoglycoside (large shapes; Fig. 4) were measured using a fluorescence-based assay (Fig. 5). The small molecules were dye labeled by conjugation to *N*-(2-propynyl) 5-fluoresceincarboxamide (Fig. 1B).¹⁰ Thus, the conjugation site employed for dye labeling is the same site that was used to conjugate the compounds to the microarray surface.

The affinities of the RNA motif–small molecule interactions studied range from 50 to 1200 nM (Fig. 5). In contrast, the affinities of each small molecule for **1** and **2** (the starting libraries) are ≥ 3000 nM, indicating that the aminoglycosides bind the randomized regions of selected RNAs. Thus, selected interactions are between the aminoglycosides and the nucleotides derived from the randomized region and not regions common to all RNA library

members. In general, there is correlation between affinity and Fitness Score, although there are a few outliers (Fig. 5).

The affinities of PAR I, AMI, and APR for selected members from **1** (internal loops) is on average approximately twofold greater than for selected members from **2**. In contrast, RIB on average binds selected hairpins with higher affinity (by ~twofold) than selected internal loops. PAR II and STR on average bind selected internal loops and hairpins with similar affinities. Previously, we reported 2DCS selections for two other aminoglycosides, 6-*N*-5-hexynoate kanamycin A and 6-*N*-5-hexynoate neamine.^{10,17,24} Both compounds bind selected internal loops more tightly than selected hairpin loops, by more than 10-fold in many cases.^{10,17,24} 2DCS selections have also been completed for neamine, kanamycin A, tobramycin, and neomycin B derivatives using a 4 × 3 nucleotide internal loop library.²⁹ The interactions selected in those studies range from 50 to 180 nM,²⁹ which is slightly higher affinity than the interactions selected in the studies reported here.

Interestingly, StARTS can predict the selectivity of an RNA motif–small molecule interaction. That is, the Fitness Scores of an RNA motif of interest for various small molecules are compared. The most selective interactions have high Fitness Scores for one small molecule and low Fitness Scores for other compounds. We analyzed the RNA motifs with the highest Fitness Scores for each azido aminoglycoside and compared them to other small molecules (Table S27). Using the predicted selectivities and measured affinities, AMI and PAR II are more selective for their corresponding internal loops (AMI IL1 and PAR II IL1). There is no clear difference in the predicted specificities or affinities of APR, PAR I, or PAR II for their selected internal loops or hairpins with the highest Sum Z-scores. In terms of selectivity, STR is more selective for STR HP1 over other hairpins than STR IL1 is over other internal loops. However, the affinity of STR for STR IL1 is approximately twofold stronger than the affinity for STR HP1. Taken together, some aminoglycosides may be more useful for targeting internal loops than hairpins and vice versa.

The selected interactions described herein are, in general, higher affinity than previously reported interactions between (unmodified) aminoglycosides and oligonucleotide mimics of the bacterial rRNA A-site.²³ For example, paromomycin, ribostamycin, apramycin, and streptomycin bind the A-site with dissociation constants of 1400, 25,000, 6300, and 94,000 nM, respectively. It remains to be seen if the interactions selected herein can be used to target non-ribosomal RNAs in biological systems although success has already been reported using selected interactions for 6-*N*-5-hexynoate kanamycin A.³¹ Factors such as cellular abundance and lifetime of the targeted RNAs are likely important. The ribosome is a privileged target because of its critically important role in cellular homeostasis, its cellular abundance (rRNA constitutes 80–90% of total RNA content), and its long lifetime.³²

The large differences in affinity between the selected RNA motifs and the bacterial A-site suggest very different binding modes. Structures of aminoglycosides bound to ribosomal particles have been refined and provide information on the specific interactions that drive molecular recognition of the A-site.^{7,33} For example, a structure of streptomycin bound to the ribosome has revealed a series of specific contacts that contribute to affinity, including an interaction with the carbonyl of the aldehyde functionality in ring II.⁷ In STR employed in our 2DCS studies, this aldehyde was removed upon reductive amination with 3-azidopropylamine. In the structure of paromomycin bound to the ribosome, a series of contacts between the ribosome and the aminoglycoside occur between the 6' hydroxyl group in ring I and the 5' hydroxyl group in ring III.⁷ In PAR I and PAR II, these functional groups are individually modified to azido groups. Thus, the specific functional groups that drive molecular recognition between paromomycin and the ribosome and PAR I and PAR II and their selected RNAs are likely different. These differences in molecular recognition suggest

that azido aminoglycosides could be used to target non-ribosomal RNAs selectively. This is bolstered by the fact that modified aminoglycosides bind the A-site with much lower affinities than the parent compounds.³⁴ It should be noted, however, that the antibacterial activity of aminoglycosides is a function of affinity and perturbation of A-site dynamics.^{4–9} It is unknown how azido aminoglycosides affect the conformational dynamics of the A-site or their selected RNAs, although it is likely that they perturb A-site dynamics to a lesser extent than their parent compounds.

The RNA motifs that were selected in these studies can be used to identify potential cellular RNA targets. Databases of the sequences and secondary structures of many non-coding RNAs are available including miRBase (microRNAs) and RNAdb (other non-coding RNAs).^{35,36} Many microRNAs have also been annotated for association with disease.³⁷ Once small molecule leads have been identified by using information such as that described herein, they can be optimized for biological activity using various approaches such as synthesis of derivatives, modular assembly, chemical similarity searching, and structure-based design.

3. Experimental section

3.1. General methods

NANOpure water was used for all experiments. All DNA oligonucleotides were purchased from Integrated DNA Technologies (IDT) and used without purification unless otherwise noted. RNA oligonucleotides were purchased from Dharmacon. The manufacturer's protocol was used in all instances. Fluorescein conjugates were quantified by using an extinction coefficient of $45,000 \text{ M}^{-1} \text{ cm}^{-1}$ at 496 nm in $1\times$ PBS, pH 7.4.³⁸

3.2. Mass spectra

Mass spectra were recorded on a 4800 plus MALDI TOF/TOF analyzer.

3.3. Preparative HPLC

Fluorescein derivatives were purified using either a reverse phase Atlantis® Prep T3 C18 5 μM column or a Sunfire Prep C18 5 μM 19 150 mm column. HPLC separations were completed using a Waters 1525 Binary HPLC Pump equipped with a Waters 2487 Dual Absorbance Detector system. A linear gradient from 0% to 100% B in A over 60 min and a flow rate of 5 mL/min were employed. (A: water + 0.1% (v/v) trifluoroacetic acid (TFA); B: methanol + 0.1% (v/v) TFA.)

3.4. Analytical HPLC

The purity of fluorescein derivatives was evaluated on a reverse phase Waters Symmetry C18 5 μM 4.6 $\times$ 150 mm column at room temperature. A flow rate of 1 mL/min and a linear gradient of 0–100% B in A over 20 min were applied. Absorbance was monitored at 220 and 254 nm.

3.5. PCR amplification of DNA templates

PCR amplification reactions were completed as previously described using the following primers: reverse primer, 5'-CCTTGCGGATCCAAT; forward primer, 5'-GGCCGAATTCTAATA CGACTCACTATAGGGAGAGGGTTTAAT.¹⁰

3.6. Construction of alkyne-displaying microarrays

Alkyne functionalized microarrays were prepared as previously described.^{12,39} Azido aminoglycosides were spotted onto alkyne-displaying microarrays in $1\times$ Spotting Solution

(10 mM Tris-HCl, pH 8.5, 100 μ M ascorbic acid, 1 mM copper sulfate, 100 μ M tris(benzyltriazolylmethyl) amine (TBTA) (dissolved in 4:1 2-butanol/dimethyl sulfoxide (DMSO)) and 10% glycerol). Five concentrations were spotted on the slide corresponding to 1500, 300, 60, 12, and 2.4 pmol of the small molecule. The slides were placed in a humidity chamber for 3 h and washed with NANOpure water. The arrays were then allowed to dry on the bench top.

3.7. RNA libraries and competitor oligonucleotides

The RNA internal loop and hairpin loop libraries were designed previously to display randomized regions in a 3×3 nucleotide internal loop pattern^{10,11} or a 6-nucleotide hairpin.²⁴ The templates for the RNA libraries were purchased from IDT in which the randomized nucleotides were custom mixed to ensure equal representation in the RNA library. The RNA motif library templates were PCR amplified as described above to provide a double stranded DNA template with a T7 promoter.

3.8. RNA transcription and purification

RNA oligonucleotides were transcribed in 1 $\times$ Transcription Buffer (40 mM Tris-HCl, pH 8.1, 1 mM spermidine, 0.001% (v/v) Triton-X 100, and 10 mM DTT),⁴⁰ 2.25 mM of each rNTP, 5 mM MgCl₂, and T7 RNA polymerase. The reaction was incubated at 37 °C overnight. The transcribed RNAs were purified on a denaturing 15% polyacrylamide gel and isolated as previously described.¹⁰ Concentrations were determined by absorbance at 260 nm and the corresponding extinction coefficient. Extinction coefficients were calculated using the HyTher server,^{41,42} which uses parameters based on extinction coefficients of RNA nearest neighbors.⁴³ Internally labeled internal loop (1) and hairpin loop (2) libraries were transcribed following the protocol described above except 1.12 mM ATP and 3 μ L of [γ -³²P] ATP were used in the transcription reaction.

3.9. RNA selection via 2DCS

Internally labeled internal loop library (1, 30 pmol), hairpin loop library (2, 30 pmol) and competitor oligonucleotides (3–8, 67.5 nmol each) were folded separately in 1 $\times$ Hybridization Buffer (HB1; 20 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (Hepes), pH 7.5, 150 mM NaCl, and 5 mM KCl) by heating at 60 °C for 5 min followed by slowly cooling to room temperature. MgCl₂ was then added to a final concentration of 1 mM. The folded oligonucleotides were mixed together in a final volume of 500 μ L. The microarray displaying serially diluted small molecules was preequilibrated with HB1 supplemented with 1 mM MgCl₂ and 40 μ g/mL bovine serum albumin (BSA) (1 $\times$ HB2) for 5 min at room temperature to prevent non-specific binding. The folded RNAs were then carefully pipetted onto the slide and evenly spread across the slide surface with a piece of parafilm. The slides were hybridized at room temperature for 45 min, and the hybridization solution was removed. The slides were washed by submersion in 1 $\times$ HB2 for 5 min and then with NANOpure water three times for 5 min each.

The arrays were exposed to a phosphorimager screen and imaged using a BioRad FX phosphorimager. The selected RNAs were then mechanically removed by excising the agarose from the surface. The RNAs were used directly in RT-PCR reactions, which were completed as previously described.¹⁰ RT-PCR products were cloned into pUC-19 using EcoRI and BamHI restriction enzyme sites. Plasmids were sequenced by Functional Biosciences, Inc.

3.10. Statistical analysis of selected RNAs via StARTS

The RNAs selected to bind a small molecule were analyzed using the RNA-Privileged Space Predictor program (RNA-PSP, v 2.0) as described previously.^{17,18} RNA-PSP compares the frequency of occurrence of a feature in the selected RNAs to its frequency of occurrence in the entire library, affording a *Z*-score. Only features that are statistically significant (95% confidence or *p* 0.05) are considered. *Z*-Scores are calculated using Eqs. 1 and 2:

$$\Phi = \frac{n_1 p_2 + n_2 p_1}{n_1 + p_1} \quad (1)$$

$$Z_{\text{abs}} = \frac{(p_1 - p_2)}{\sqrt{\Phi(1 - \Phi) \left(\frac{1}{n_1} + \frac{1}{n_2} \right)}} \quad (2)$$

where Φ is the pooled sample proportion; n_1 is the number of selected RNAs from **1** or **2**; n_2 is the size of the starting library (**1** or **2**; 4096); p_1 is the observed proportion of selected RNAs displaying the trend; and p_2 is the observed proportion of **1** or **2** (entire library) displaying the trend.

Each RNA can display more than one statistically significant feature. The corresponding *Z*-scores of these features are summed to afford a Sum *Z*-score. The highest Sum *Z*-score for each selection was used to normalize all possible RNA motif–small molecule interactions in the selection, affording a Fitness Score.

3.11. Binding affinity measurements

Dissociation constants were determined using a fluorescence-based assay as described previously.¹⁰ Briefly, a selected RNA was folded in 1× HB1 supplemented with 40 µg/mL BSA by heating at 60 °C for 5 min followed by slowly cooling to room temperature. MgCl₂ was then added to a final concentration of 1 mM. Fluorescently labeled ligand was added to a final concentration of 50 nM. Serial dilutions were then completed in 1× HB2 supplemented with 50 nM fluorescently labeled ligand. The solutions were incubated for 30 min at room temperature and then transferred to a half-area 96-well black plate (Corning, Inc.). Fluorescence intensity was measured on a Bio-Tek FLx-800 plate reader. The change in fluorescence intensity as a function of RNA concentration was fit to Eq. 3:^{10,12}

$$I = I_0 + 0.5 \Delta \epsilon \left(([\text{FL}]_0 + [\text{RNA}]_0 + K_t) - \left(([\text{FL}]_0 + [\text{RNA}]_0 + K_t)^2 - 4 [\text{FL}]_0 [\text{RNA}]_0 \right)^{0.5} \right) \quad (3)$$

3.12. Synthesis of fluorescently labeled aminoglycosides

N-(2-Propynyl) 5-fluoresceincarboxamide (FI) was synthesized as previously reported.¹⁸ A 3 µmol sample of FI in dimethyl sulfoxide (DMSO) was added to a solution containing 1 µmol of the corresponding azido aminoglycoside, 10 µmol of copper sulfate, 20 µmol of freshly prepared ascorbic acid in water, and 300 nmol of TBTA (dissolved in DMSO). The final volume was brought to 2.5 mL with *N,N*-dimethylformamide (DMF). The reaction mixture was transferred to a microwave reaction vessel and placed in Emry's Optimizer microwave system (Biotage). The reaction was maintained at 110 °C for 4 h with stirring. The product was purified by preparative HPLC and characterized by MALDI mass spectrometry. Purity was assessed by analytical HPLC. All fluorescein conjugates were 95% pure.

PAR I-Fl: t_R = 41 min (Atlantis®Prep T3 C18 5 μ M); MALDI: $M+H^+$ calculated: 1054; $M+H^+$ observed: 1054; 24% yield. PAR II-Fl: t_R = 31 min (SunfirePrep C18 5 μ M); MALDI: $M+H^+$ calculated, 1054; $M+H^+$ observed: 1054; 50% yield. RIB-Fl: t_R = 34 min (SunfirePrep C18 5 μ M); MALDI: $M+H^+$ calculated: 893; $M+H^+$ observed: 894; 28% yield. AMI-Fl: t_R = 34 min (SunfirePrep C18 5 μ M); MALDI: $M+H^+$ calculated: 1024; $M+H^+$ observed: 1024; 15% yield. APR-Fl: t_R = 33 min (SunfirePrep C18 5 μ M); MALDI: $M+H^+$ calculated: 978; $M+H^+$ observed 978; 32% yield. STR-Fl: t_R = 29 min (Atlantis®Prep T3 C18 5 μ M); MALDI: $M+H^+$ calculated: 1080; $M+H^+$ observed: 1080; 35% yield.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

2DCS	two-dimensional combinatorial screening
AMI	6 azido amikacin
AmG	aminoglycoside
APR	6 azido apramycin
HB1	1× Hybridization Buffer 1
HB2	1× Hybridization Buffer 2
HP	hairpin loop
IL	internal loop
PAR I	6 azido paromomycin
PAR II	5 azido paromomycin
RIB	5 azido ribostamycin
RNA-PSP	RNA-privileged space predictor
RT	reverse transcriptase
RT-PCR	reverse transcriptase-polymerase chain reaction
STARTS	structure–activity relationship through sequencing
STR	3-azido streptomycin
TBTA	tris(benzyltriazolylmethyl) amine

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Figure 1.

(A) Chemical structures of azido aminoglycosides used in this study: PAR I (6 azido paromomycin), PAR II (5 azido paromomycin), RIB (5 azido ribostamycin), AMI (6 azido amikacin), APR (6 azido apramycin), and STR (3-azido streptomycin). (B) Schematic for immobilization of azide functionalized aminoglycosides onto alkyne-displaying microarrays or conjugation to fluorescein (green ball), both via Huisgen dipolar cycloaddition reactions. AmG refers to aminoglycoside.

Figure 2.

Secondary structures of oligonucleotides used in this study. Compound **1** is a 3×3 nucleotide internal loop library while **2** is a 6-nucleotide hairpin library. Oligonucleotides **3–8** are competitor oligonucleotides used to constrain the selected interactions to the randomized regions (N's) of **1** and **2**.

Figure 3.

(A) Microarray surface after hybridization with radioactively labeled **1** and **2** and competitor oligonucleotides **3–8**. Circles indicate ligand bound RNAs that were excised. The moles of small molecule that were spotted are 1500, 300, 60, 12, and 2.4 pmol. (B) Dose response for binding of **1** and **2** to azido aminoglycosides displaying on a microarray. Compounds **1** and **2** were present in equimolar amounts. Competitor oligonucleotides **3–8** (Fig. 2) were present in threefold excess over the moles of compounds delivered to the slide surface and >1000-fold excess over **1** and **2**. (C) Representative gel image of RT-PCR amplification of selected RNAs. A '+' indicates that reverse transcriptase was added while a '-' indicates that it was absent.

Figure 4.

StARTS analysis of the selected RNAs derived from **1** or **2** for binding azido aminoglycosides. (A) Fitness Scores for the selected RNA motifs from **1** and azido aminoglycosides. The affinities of the five RNA motifs with highest Fitness Scores (indicated by larger circles) and the corresponding aminoglycoside were measured (Fig. 5). (B) Fitness Scores for selected hairpins (derived from **2**) and the corresponding aminoglycoside. The affinities of the five RNA motifs with the highest Fitness Scores (indicated by larger squares) and the corresponding aminoglycoside were measured (Fig. 5).

Figure 5.

Predicted secondary structures of selected RNA motifs with highest Fitness Scores and their corresponding affinities and Fitness Scores. The secondary structures were predicted by the RNA structure program.⁴⁴ The nomenclature for the loops refers to the ligand for which the loops were selected followed by either internal loop (IL) or hairpin (HP) and a number. The values directly below the loop identifier are the binding affinities for the corresponding RNA-small molecule complexes (nM). Fitness Scores are indicated below affinities. (A) A subset of the internal loops and hairpins selected to bind PAR I and the corresponding affinities and Fitness Scores; (B) a subset of the internal loops and hairpins selected to bind PAR II and the corresponding affinities and Fitness Scores; (C) a subset of the internal loops, bulge, and hairpins selected to bind RIB and the corresponding affinities and Fitness Scores; (D) a subset of the internal loops and hairpins selected to bind AMI and the corresponding affinities and Fitness Scores; (E) a subset of the internal loops, bulge, and hairpins selected to bind APR and the corresponding affinities and Fitness Scores; and (F) a subset of the internal loops and hairpins selected to bind STR and the corresponding affinities and Fitness Scores.