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Aminoglycoside array for the high-throughput analysis of small molecule–RNA interactions

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Abstract—Aminoglycoside-based oligosaccharides were bound to microtiter plates using noncovalent display with hybridization confirmed by mass spectrometry. Recognition of the saccharides by a model of the prokaryotic 16*S* aminoacyl site of the ribosomal decoding region was then determined. Tobramycin binding constants that correlated with surface plasmon resonance values for the model were effectively measured and novel means for binding aminoglycoside-based members were discovered for application to future aminoglycoside libraries.

While DNA is the genetic library or vault of the cell, RNA is its active messenger.[1,2] The RNA molecules are responsible for their own translation and fidelity and many additional catalytic functions that are still being understood. In addition, many new targets identified from genome research are proteins with complex post-translational modifications (e.g., glycosylation) and access to these biomolecules becomes a new challenge. Targeting the RNA responsible for the corresponding translational process is thus considered to be an alternative strategy for drug discovery.[3–5] Despite its therapeutic benefits, few methods are available for the high-throughput analysis of small molecule–RNA interactions.[6–8] As such, we sought to design a high-throughput assay for the rapid discovery of RNA–small molecule interactions. While RNA–small molecule interactions have been studied in solution using a microtiter plate setting,[8] we sought to apply the noncovalent carbohydrate array format that we have previously used to observe oligosaccharide–protein interactions in a high-throughput manner.[9,10]

In order to develop this method, we chose to study the well-characterized interactions of aminoglycosides with RNA. Aminoglycosides constitute a family of antibiotics that derive their antibiotic activity through binding to the major groove of the prokaryotic 16*S* aminoacyl (A) site ribosomal decoding region (Fig. 1).[11–14] This binding, mediated by recognition of an adenine bulge, leads to an altered, energetically favorable conformation in the A-site RNA resulting in miscoding and truncation of proteins (Fig. 2A). As the interactions between aminoglycosides and an rRNA A-site model system have been well characterized,[12] we used this to screen a library of aminoglycosides (**2**–**12**; Fig. 3) in hopes of finding analogs with novel architectures and binding.

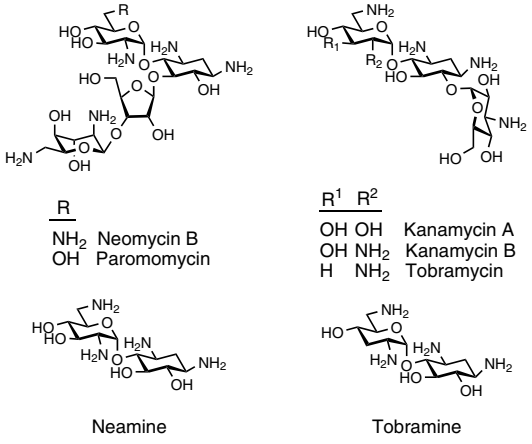

Figure 1. Examples of aminoglycosides with the top two rings of neomycin and tobramycin (neamine and tobramine, respectively).

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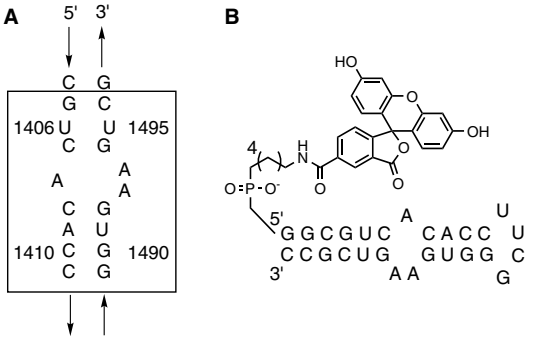

Figure 2. The 16*S* ribosomal RNA A-site (A) and the fluorescein-labeled A-site rRNA model used in binding studies (B).

Galactosyllipid **1**[9] was designed as a negative control as alteration in the top ring has been illustrated to give loss in RNA binding.[15] Compound **2** is a tobramycin analog with replacement of the C′6 hydroxyl on ring III with an amine to which the 15 carbon tether is attached. Compounds **10** and **11** both contain tobramine with different substituents and **12** is the neamine core. The remaining library members, **3**–**9**, contain di- and tri-saccharides of aminated glucose and galactose that were created using Optimer one-pot oligosaccharide synthesis.[16] Other compounds were prepared following strategies described previously.[17,18] After incubation of the library in the microtiter plate, binding to solid phase was verified by MALDI-FTMS analysis.[19,20]

As the interaction between tobramycin **2** and the model sequence of RNA (Fig. 2B) has been characterized,[12] this member was used in the design of the RNA–aminoglycoside assay. Initial attempts to use biotinylated RNA were unsuccessful as the wash conditions were too harsh for the RNA–carbohydrate interactions. Therefore, a 5′-fluorescein-labeled RNA (Fig. 2B) was used for direct fluorescence detection.[21] Increasing concentrations of compound **2** gave the expected logarithmic binding curve for the RNA model sequence (Fig. 4). By plotting the fluorescence against the fluorescence multiplied by the inverse concentration of tobramycin (inset, Fig. 4), a K_d value of $1.9 \pm 0.3\,\mu M$ was determined. This binding affinity is very close to the reported value of $1.50\,\mu M$ as determined by surface plasmon resonance (SPR).[22] Moreover, this method was able to measure all concentrations simultaneously in a very short time period and is much less expensive than standard SPR.

Interactions of the aminoglycoside library with the RNA were then analyzed (Fig. 5). As expected, no fluorescence over background was observed for galactosyllipid **1**. Also, tobramycin, which was expected to most efficiently bind the RNA sequence based on previously determined K_ds,[22] did in fact afford the highest fluorescence intensity. Compounds **3** through **5**, which contain 6-amino-6-deoxy-D-galactose linked to 2-amino-2-deoxy-D-glucose with variation in the linkage between the anomeric position of galactose and glucose, all showed poor fluorescence intensity when compared to tobramycin. Addition of a third monosaccharide did

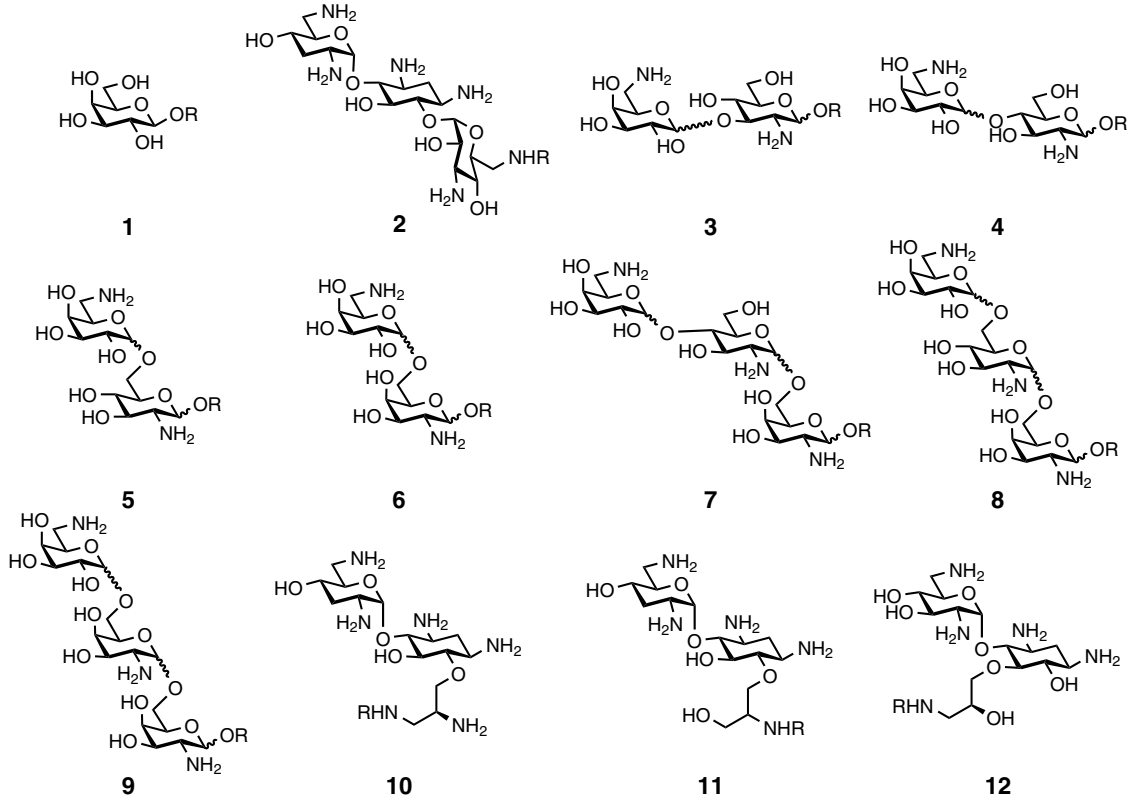

Figure 3. All compounds contained a 15 carbon, fully saturated hydrocarbon tether for hydrophobic attachment to high binding, polystyrene microtiter plates. Attachment was either through an ether (**1**, **3**–**9**) or amine (**2**, **10**–**12**). R=$C_{15}H_{31}$.

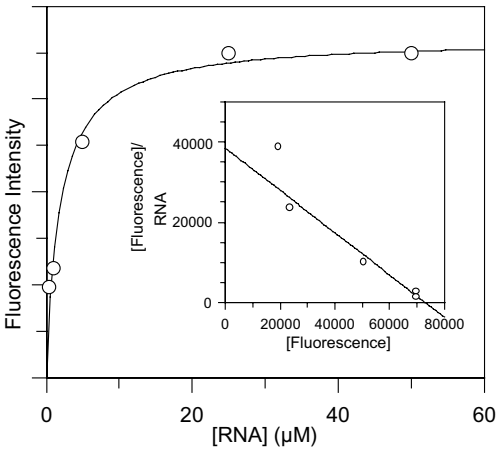

Figure 4. Concentration dependent binding of A-site rRNA to bound tobramycin **2**. K_d was determined by plotting the fluorescence against the fluorescence multiplied by the inverse concentration of RNA bound.

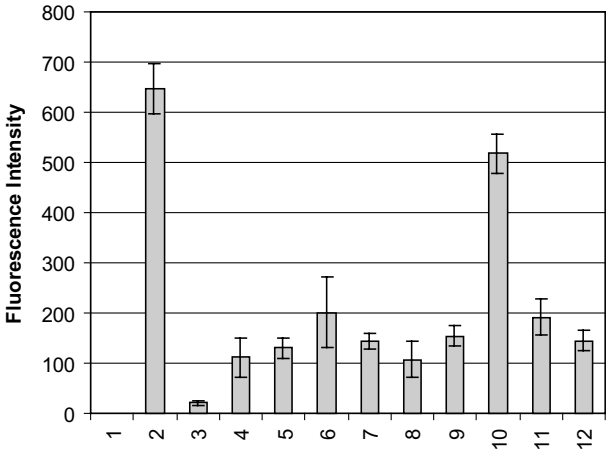

Figure 5. Fluorescence of A-site rRNA bound to compounds **1** through **12**.

little to affect the fluorescence intensity as illustrated in the fluorescence intensities for compounds **7**–**9** as compared to **4**–**6**.

The remaining library members, **10** through **12**, contain architectures that have been well characterized in relation to binding to the A-site rRNA.[23] Compounds **10** and **11** contain the tobramine core with differing lipid tethers and compound **12** includes neamine. Of these three, compound **10** is of greatest note as it shows 80% of the relative fluorescence of tobramycin control **2** and has the strongest RNA signal of any library member. Neamine conjugate **12** showed only 22% fluorescence intensity relative to compound **2**, roughly equivalent to that of tobramine analog **11**. The binding affinities of tobramine and neamine alone are very similar (6.40 and 7.80 μM, respectively), illustrating that this increased binding for compound **10** must be a linker effect.[22]

Features that could be responsible for this include degree of amination and position of free amines and hydroxyls on the linker. For this first case, the presence of a second amine on compound **10** may be an important binding factor as increased amination is known to lower the K_ds of aminoglycoside analogs.[24] However, the positions of the different functionalities may also play a role in mediating this binding. Tobramycin is known to interact with the G1405=C1496 pair of the A-site rRNA model through O′2 and N′3 of ring III.[12] Compound **10** contains a primary amine at the β position while compound **11** displays a secondary amine with uncertain stereochemistry. As primary amines on aminoglycosides tend to have pK_as between 7 and 8,[13] this primary amine should act as a better hydrogen bond donor for interaction with G1405 than the corresponding nitrogen in compound **11** giving improved binding. In addition, the γ nitrogen of **10** should still be a better donor than the hydroxyl group in **11**. In a separate study, we found that the activity of a tobramycin analog where the functional groups at C′2 and C′3 of ring III were switched remained the same. Considering the interaction of aminoglycosides with RNA,[12] compound **10** may be a mimic of this analog. All these factors, though, may be responsible for the increased retention of RNA to analog **10**. Attachment of this functionalized linker to the tobramine core may lead to a simplified backbone capable of recognizing RNA specifically or circumventing bacterial resistance as has been successfully shown before with a novel aminoglycoside-based molecule.[25]

In summary, an approach for the rapid screening of libraries against RNA has been developed using noncovalent attachment of the small molecule to the surface of a microtiter plate and analysis of RNA binding in solution. This assay is both facile and highly reproducible and was applied to the analysis of a small aminoglycoside library against a model of the A-site ribosomal RNA of *E. coli*. Analysis of the library gave insight into the importance of the linkages between the monosaccharides of the compounds as well as the unimportance of a third monosaccharide, at least in these molecules. Compound **2** complexed with the fluorescent RNA A-site in microtiter plates may serve as a platform for a competitive high-throughput screening to identify new small molecules to target this particular RNA sequence. Such a strategy should also be useful in the high-throughput analysis of the effect of various modifications to aminoglycoside structure on electrostatic interactions.[3,26] This strategy should in principle be applicable to other RNA targets as well. It is also of note that addition of a free amine to the linker tether greatly increased the RNA retention. This observation may lead to improved antibiotic therapeutics if applied to future aminoglycoside analogs.

Acknowledgements

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References and notes

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18. Physical data is consistent with expected results.
19. Compounds (10 nM in MeOH) were added to the microtiter plate wells, MeOH was evaporated, and wells were washed with 50 mM Tris–HCl, pH 7.5/150 mM NaCl three times. MeOH was then added to the wells and the solution was submitted for MALDI-FTMS and ESI analysis.
20. MS of saccharides **1**–**12**, after incubation in microtiter plate: MALDI-TOF: compound **1**: $[M + Na]^+$, 413.2870; compound **2**: $[M + Na]^+$, 699.5005; compound **3**: $[M + Na]^+$, 573.3716; compound **4**: $[M + Na]^+$, 573.3719; compound **5**: $[M + Na]^+$, 573.3726; compound **6**: $[M + Na]^+$, 573.3722; compound **9**: $[M + Na]^+$, 734.4406; compound **10**: $[M + Na]^+$, 612.4648; compound **12**: $[M + Na]^+$, 628.4616. ESI: compound **7**: $[M + Na]^+$, 733; compound **8**: $[M + Na]^+$, 733; compound **11**: $[M + H]^+$, 590.
21. Compound **2** was dissolved in MeOH and added to the microtiter plate. After evaporation of the MeOH, wells were washed with 50 mM Tris–HCl, pH 7.5/150 mM NaCl (buffer A). Wells were then blocked with buffer A supplemented with 4 mM $MgCl_2$ and 1% BSA (buffer B) over an hour. The solvent was then removed and the A-site rRNA sequence (5 μM) was added. After 5 min, the RNA was tapped out and the plate was washed with buffer B (100 μL) one time. Fresh buffer B (100 μL) was then added and fluorescence was read at 535 nm. RNA sequence: 5′-fluorescein-GGCGUCACACCUUCGGGU-GAAGUCGCC-3′ from Dharmacon RNA Technologies.
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