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Nonionic Side Chains Modulate the Affinity and Specificity of Binding between Functionalized Polyamines and Structured RNA

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This Communication introduces a class of molecules with characteristics for specific recognition of folded RNA structures. The wealth of structural information available on RNA reveals the complexity and diversity of the folded structures that RNA can adopt to create protein-binding sites or to perform catalytic functions.[1] New molecules that recognize a three-dimensional RNA structure and bind with high affinity and specificity could ultimately evolve into new types of drugs that exert their biological effects by targeting RNA.[2]

Cationic aminoglycosides,[3] polypeptides,[4] and peptidomimetics[5] are well-known classes of organic molecules that bind RNA. While cationic charge is important for high-affinity binding to RNA, too much electrostatic attraction typically decreases binding specificity for one RNA over others.[6] Developing organic molecules that can bind RNA with high affinity *and* specificity is a challenge that must be overcome for RNA to be considered a viable drug target.

We are exploring whether side-chain-functionalized polyamines can bind specifically to folded RNA structures. Polyamines with nonionic side chains should sterically shield the cationic charges in the backbone and reduce their capacity to engage in nonspecific interactions. Furthermore, we are examining whether the side chains can be optimized to direct binding specificity. To examine these ideas, polyamine trimers (Figure 1A) have been synthesized, and their binding to two different folded RNAs associated with HIV, TAR, and RRE (Figure 1B,C), has been studied. TAR and RRE represent two RNA secondary structures with similar features: both have a stem and loop with bulges as the sites for protein binding.[7] The results presented demonstrate that functionalized polyamines can be readily synthesized, and that binding affinity and specificity are sensitive to alteration of the side chains.

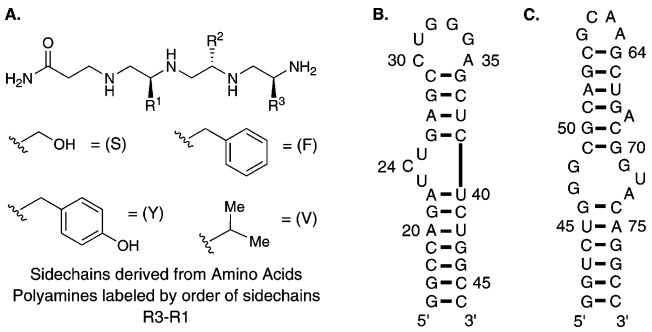
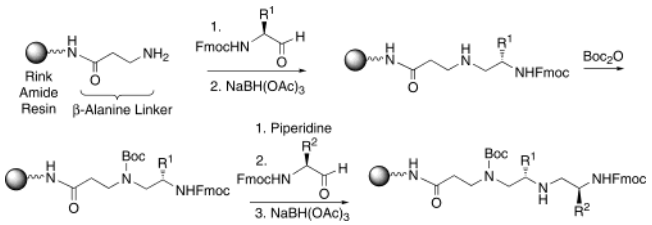


Figure 1. (A) Polyamine scaffold and side chains. (B) Secondary structure of TAR RNA. (C) Secondary structure of RRE IIB RNA.

Scheme 1. Solid Phase Synthesis of Polyamines



Polyamines were prepared using a series of reductive alkylations between primary amines on solid support and Fmoc-protected α-amino aldehydes derived from phenylalanine, tyrosine, serine, or valine (Scheme 1). Sodium triacetoxyborohydride was used to reduce the imine to the secondary amine, which was then Boc protected. Polyamine trimers were cleaved under acidic conditions and purified by HPLC to give the tetra-trifluoroacetate salt.[8]

Six polyamines with different side chain sequences have been synthesized: YYY, YSY, YVV, FFF, FSF, and FSS. Two different RNA footprinting methods were used to view interactions at each base, allowing the identification of binding sites and extraction of apparent K_D's for those sites. Both assays were performed under high salt conditions (100 mM NaCl) to limit nonspecific electrostatic effects and mimic physiological conditions. The first assay involved competition with terbium cations, which are known to cleave single-stranded regions of RNA.[9] Increasing concentrations of polyamine were incubated with ^{32}P-labeled RNA in the presence of terbium, and the cleavage products were analyzed by denaturing PAGE. When binding of the polyamine to the RNA occurred, such as in the case of YYY, the fraction of RNA cleaved at the binding site decreased with increasing polyamine concentration (Figure 2). This decrease was fit to a simple single-site binding curve (Figure 3A,B). For polyamines FFF, FSS, and FSF, either the curve did not fully saturate under the concentration range tested, indicating weak binding, or there was no effect on cleavage of the RNA.

The second RNA footprinting assay probed the effect of the polyamines on magnesium-catalyzed hydrolytic cleavage of the phosphodiester backbone. The rate of this cleavage is determined by the RNA's ability to access conformations that favor "in-line attack" of the 2′-hydroxyl on the phosphorus.[10] Typically, binding of ligands causes a change in the conformation of the RNA, leading to a change in the rate of cleavage at the binding sites. Most of the polyamines had no effect on the cleavage or caused a nonspecific decrease at millimolar concentrations. However, polyamines YYY and YSY caused an increase in cleavage, not the typical decrease. For YYY this increase saturated, and the data were fit to a single-site binding curve, from which a K_D was extracted.[8]

As a control, the terbium assay was performed on a YYY tripeptide (a neutral analog) to determine if the side chains alone were responsible for binding. Spermine, a polyamine with no side chains, was also tested to determine whether a protonated backbone was sufficient. Over the same concentrations as the synthesized polyamines, no effect on cleavage was seen, suggesting that the combination of side chains and a charged backbone is necessary for binding.

The data obtained show that binding constants for the polyamines can range from moderate binding (4 μM) to very weak binding, depending on the side chains present. The results indicate that the nitrogens of the backbone do not dominate binding interactions with

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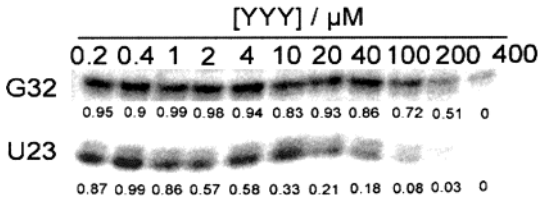

Figure 2. Portions of a 20% denaturing polyacrylamide gel showing the cleavage products for G32 (loop) and U23 (bulge) of TAR. The intensity of each band was divided by the total activity in the lane to account for differences in loading. The numbers below the bands are the normalized fraction cleaved (1 = maximum cleavage, 0 = minimum cleavage).

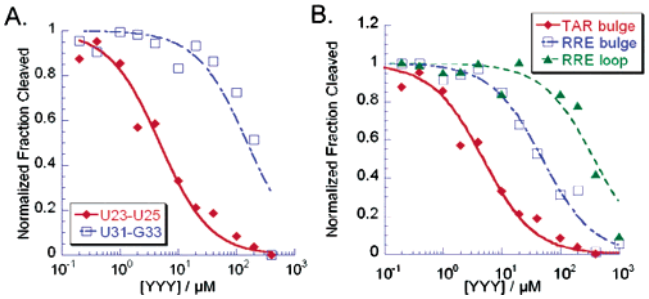

Figure 3. Binding curves for (A) YYY vs TAR, Tb cleavage, bulge (U23−U25) and loop (U31−G33) regions; (B) YYY vs TAR, Tb cleavage, bulge region, and YYY vs RRE, Tb cleavage, bulge and loop regions.

the RNA. Also, binding affinity is not simply a function of the number of aromatic groups (compare YVV to YSY in the terbium data). Significant selectivity is demonstrated in the case of polyamine YYY. This polyamine binds the bulge of TAR preferentially over the loop of TAR (Figure 3A), and over the loop and bulge of RRE (Figure 3B), by 1 order of magnitude. YYY is clearly better suited to binding the bulge of TAR than any of the other polyamines, and is not as well suited to binding the other RNA motifs studied. The terbium assay was performed at pH 6.5, and the "in-line" assay was performed at pH 8.0, spanning relevant physiological pH ranges. The dissociation constants obtained from both methods of footprinting were consistent, suggesting that binding tolerates small changes in pH and salt concentration. Based on binding curves that were fit to determine Hill coefficients, the stoichiometry of YYY binding to the TAR bulge is likely to be 1:1.[8] Other polyamines, such as YVV, showed no preference for TAR over RRE, suggesting that the preference of YYY for TAR is not caused by a more accessible structure in the TAR bulge.

Generally, the binding of the polyamines was weak and nonspecific; however, two polyamines, YYY and YSY, targeted one area of TAR RNA selectively. For these two polyamines, the binding affinity is still much too weak and the specificity is not sufficient for use as an active drug. Furthermore, it is possible that there are other weak binding sites on the RNAs that we cannot measure with these techniques.[8] However, considering the simplicity of the system and the fact that the sequence was selected randomly,

Table 1. Dissociation Constants for Polyamines to the Bulge of TAR and Internal Loop of RRE

polyamine[a]	RNA	Tb K_D (μM)	in-line K_D (μM)
YYY	TAR	4.8 ± 0.6	4.1 ± 0.6
YYY	RRE	50 ± 7	54 ± 7
YSY	TAR	>300	>150
YSY	RRE	250 ± 50	nm
YVV	TAR	100 ± 23	nm
YVV	RRE	85 ± 18	nm

[a] See Figure 1A for structures of side chains. Order of letters corresponds to order of side chains (R^3 to R^1) on polyamine backbone as illustrated in Figure 1A. nm = not measurable.

the polyamine displays activity that is very encouraging. The nature of the synthesis allows a combinatorial approach to be adopted so that optimal binding can potentially be selected from a wide variety of polyamines.

In conclusion, a new class of functionalized polyamines has been synthesized, and their study has revealed several promising characteristics for development into RNA-binding molecules. Further work will focus on optimizing the polyamines to bind tightly and specifically to target RNAs.

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Supporting Information Available: Details for synthesis, purification, and characterization of polyamines; conditions for footprinting assays and binding data. This material is available free of charge via the Internet at http://pubs.acs.org.

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