Designing Novel RNA Binders

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In memory of Jacqueline Libman

Abstract: RNA molecules play key roles in vital biological processes and are attractive targets for therapeutic intervention. The successful design of small organic molecules as specific RNA binders requires intimate knowledge of RNA structure, folding and recognition. While far from being comprehensive, our understanding of the modes in which RNA is recognized by various ligands has advanced significantly. Investigation of the interactions between aminoglycoside antibiotics, a family of polycationic pseudo-oligosaccharides, and various RNA molecules has unraveled certain RNA–ligand recognition rules. Electrostatic interactions have been found to be of critical importance for RNA binding, and a recognition model suggesting three-dimensional electrostatic complementarity has been proposed. Issues of binding specificity and factors that have to be considered in designing new RNA binders are discussed.

Keywords: aminoglycosides · antibiotics · electrostatic interactions · molecular recognition · RNA

Introduction

Despite the central role RNA plays in the replication of bacteria and viruses, drugs targeting pivotal RNA sites of these pathogens are scarce. In principle, RNA molecules are promising molecular hosts because of their distinctive architecture of sophisticated secondary and tertiary structures.[1] Ideally, one would like to identify recognition rules and well-defined binding motifs, and utilize that knowledge for the design of specific RNA binders. While our understanding of RNA structure and folding, as well as the modes in which RNA is recognized by other ligands, is far from being comprehensive, significant progress has been made in the last decade.[2, 3] RNA is taking a unique place among biomolecules with respect to the challenges and the promise it presents.

The purpose of this article is to discuss recent advances in the area of RNA recognition, focusing on RNA–aminoglycoside interactions. These natural products have been shown to interact with a variety of RNA molecules. In contrast to classical intercalators, which typically bind to both DNA and RNA,[4] aminoglycosides show a promising preference for RNA binding by electrostatic, nonintercalative interactions. We view RNA–aminoglycoside binding as a paradigm that allows us to learn how RNA and small charged organic molecules interact. The knowledge thus gained can be translated into the design and synthesis of ligands that can target specific RNA molecules. In this Concept article, we briefly review the background and summarize recent results in the area of RNA–aminoglycoside interaction. We discuss issues of specificity and factors that must be considered in designing new RNA binders.

Discussion

Aminoglycoside antibiotics: The search for novel organic molecules as RNA binders was inspired by early observations indicating the ability of certain natural products to mediate translation.[5–7] RNA is a major constituent of the ribosome, where protein biosynthesis is regulated by a complicated interplay of transfer RNA (tRNA), messenger RNA (mRNA), ribosomal RNA (rRNA), and ribosomal proteins as well as cofactors. A variety of aminoglycoside antibiotics, 2-deoxystreptamine-containing amino oligosaccharides, are known to interfere with ribosomal function in prokaryotes.[8, 9] They bind to the decoding region (A site) of the 16S rRNA of the 30S subunit, thus interfering with the fidelity of translation and translocation by decreasing the dissociation rate of cognate and semicognate aminoacylated tRNA from the ribosome.[10] This ultimately disrupts bacterial protein biosynthesis. Despite the established bactericidal properties of aminoglycoside antibiotics, their therapeutic use is limited, as internal administration of aminoglycoside antibiotics at high doses results in clinical side effects (e.g., nephro- and ototoxicity associated with irreversible hearing loss).[11]

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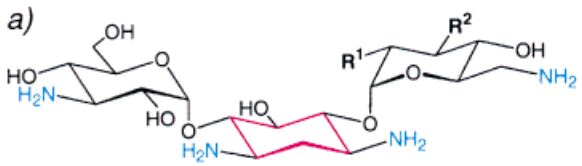
More recently, aminoglycoside antibiotics have been shown to interact with a variety of other natural RNA molecules. A number of them effectively inhibit splicing of Group I introns,[12] the hammerhead ribozyme,[13, 14] and the human hepatitis delta virus ribozyme.[15, 16] Certain aminoglycoside antibiotics inhibit HIV-1 replication by blocking the interactions of the HIV essential proteins Tat and Rev with their corresponding RNA recognition elements.[17, 18]

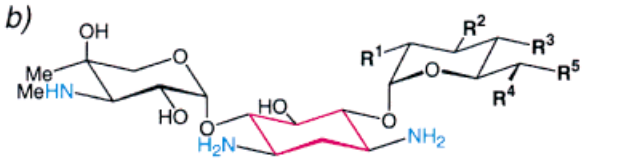
Representative examples of aminoglycoside antibiotics and their in vitro activity as inhibitors of translation, self-splicing, and Rev–RRE (Rev-response element) binding are shown in Figure 1. The importance of the number of amino groups for RNA binding and inhibition is revealed by several structure–activity relationships. For example, changing an amino group to a hydroxyl group in kanamycin B versus kanamycin A (Figure 1a) and neomycin B versus paromomycin (Figure 1c) practically eliminates inhibitory activity in the self-splicing and Rev–RRE assays. A similar trend is observed in the gentamycin family (Figure 1b). Electrostatic interactions are likely to play a crucial role in RNA binding. Yet this recognition phenomenon is far more sophisticated, as simple polyamines (e.g., spermine), other aminoglycosides (e.g., apramycin, Figure 1d) and other structurally unrelated antibiotics (e.g., viomycin) that possess comparable numbers of amino groups are not active. As evident from the structure–activity relationship shown in Figure 1, the sugar hydroxyls also have a significant effect on the RNA-binding capability of the aminoglycosides. Kanamycin B is twentyfold less active than tobramycin (its 3′-deoxy derivative) in inhibiting self-splicing of Group I introns, and a similar trend has been found in the inhibition of Rev–RRE binding. Furthermore, while certain aminoglycosides are very active in all assays (e.g., neomycin B), this trend is not universal. For instance, paromomycin, which is a poor self-splicing and Rev–RRE inhibitor, is a reasonable translation inhibitor.

Stimulated by these intriguing observations, we have embarked on a research program aiming at the fundamental understanding of RNA–aminoglycoside interactions. The following discussion summarizes our current understanding in this area. We discuss the role of electrostatic interactions in RNA binding, the role played by the different functional groups, and issues of specificity. As this field has been attracting a considerable amount of attention lately, it is likely that our knowledge of RNA–aminoglycoside recognition will continue to advance rapidly.

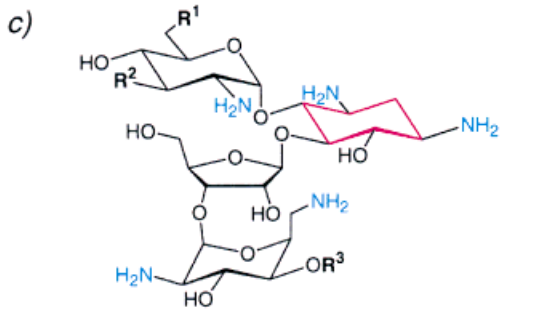
Deciphering RNA–aminoglycoside interactions: Designing an RNA-binding molecule in the absence of sufficient structural information on the receptor site and on the functional groups involved in recognition is a challenging task.[19] Nevertheless, we can elucidate certain recognition rules by examining the structure–activity relationship (SAR) in RNA–aminoglycoside binding. Since aminoglycosides seem to recognize their RNA targets by similar structural motifs rather than by sequence, we can choose an RNA binding assay and deduce recognition rules which may have



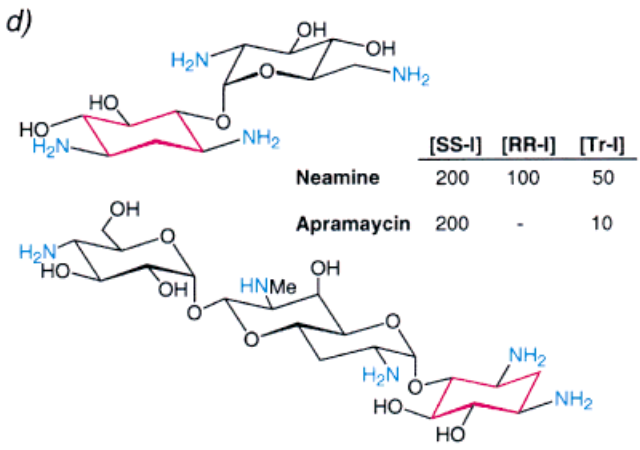
	R^1	R^2	[SS-I]	[RR-I]	[Tr-I]
Kanamycin A	OH	OH	$>2 \times 10^3$	> 100	5
Kanamycin B	NH_2	OH	10	100	1
Tobramycin	NH_2	H	0.5	10	1



	R^1	R^2	R^3	R^4	R^5	[SS-I]	[Tr-I]
Gentamycin C_1	NH_2	H	H	CH_3	$NHCH_3$	1	1
Gentamycin B	OH	OH	OH	H	NH_2	10	20
G-418	NH_2	OH	OH	CH_3	OH	$>2 \times 10^3$	20



	R^1	R^2	R^3	[SS-I]	[RR-I]	[Tr-I]
Neomycin B	NH_2	OH	H	0.5	1	0.5
Paromomycin	OH	OH	H	100	>100	10
Lividomycin A	OH	H	Man	500	10	10



	[SS-I]	[RR-I]	[Tr-I]
Neamine	200	100	50
Apramaycin	200	-	10

Figure 1. Structure–activity relationships of natural aminoglycoside antibiotics. The inhibition of self-splicing of group I intron (**SS-I**) is given as IC_{50} values in μM;[12] inhibition of Rev–RRE binding (**RR-I**) as IC_{90} values in μM;[18] inhibition of in vitro translation (**Tr-I**) as IC_{50} values in μM.[12c] The amino groups and the 2-deoxystreptamine (2-DOS) core are highlighted.

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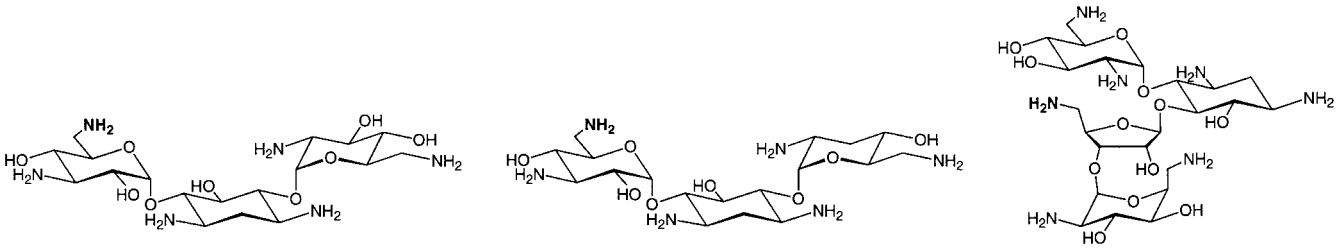

Figure 2. Structures of synthetic amino-aminoglycosides. The amino groups that replace the hydroxyl groups in the corresponding natural products are shown in bold.[23]

universal validity. Most of our studies have been carried out with the hammerhead and the *Tetrahymena* ribozymes. Following the modulation of the catalytic activity of such functional RNA molecules by aminoglycosides and their derivatives is a fast and efficient way of assessing RNA binding, particularly when comparative studies are conducted. It has to be kept in mind, though, that strong binding at a remote site that does not influence catalytic activity might not be revealed.

The reported structure–activity relationships for natural aminoglycosides (Figure 1) suggest that the overall charge density presented by the aminoglycosides toward the RNA host is likely to be important for binding. Hence, aminoglycosides containing four amino groups (e.g., kanamycin A) show very little RNA binding capability, while the most active derivatives contain five or six amino groups (e.g., tobramycin and neomycin B, respectively).[12, 14, 18] At pH 7.0, these amino groups are predominantly charged,[20–22] suggesting an important role for strong electrostatic interactions in RNA–aminoglycoside binding.

We have recently reported the design, synthesis, and hammerhead ribozyme inhibitory activity of novel amino-aminoglycosides derived from kanamycin A, tobramycin, and neomycin B (Figure 2).[23] We have demonstrated that substituting a hydroxyl with an amino group can convert a very poor RNA binder, such as kanamycin A, to a reasonably strong one. Thus, 6′′-amino-6′′-deoxykanamycin A is as effective as kanamycin B, a natural product containing five amino groups. Modifying a stronger RNA binder such as tobramycin further enhances its affinity to RNA. Thus, 6′′-amino-6′′-deoxytobramycin is approximately five times more effective than the parent tobramycin as a ribozyme inhibitor. Even the binding affinity of neomycin B, one of the strongest RNA binders, can be further enhanced by converting it to 5′′-amino-5′′-deoxyneomycin B. These results suggest that increasing the overall charge of a ligand is an important mechanism for increasing RNA affinity. As illustrated below, however, this general view needs to be fine-tuned in order to explain differences in RNA binding observed among related aminoglycoside antibiotics.

Tobramycin binds a number of natural RNAs with higher affinity than kanamycin B (Figure 1),[12, 18, 23] although both compounds contain five amino groups. The only difference between the two antibiotics is an additional hydroxyl group at the 3′-position in kanamycin B. Is the presence of hydroxyl groups impeding strong RNA binding? We have studied a series of deoxygenated tobramycin derivatives in which a single hydroxyl group is removed (one at a time) while all the other functional groups are kept intact.[14] The derivatives studied are shown in Figures 1 and 3 and include kanamycin B, tobramycin, 2′′-deoxytobramycin, 4′′-deoxytobramycin, 6′′-deoxytobramycin and 4′-deoxytobramycin (dibekacin). Among these derivatives, the most potent ribozyme inhibitors

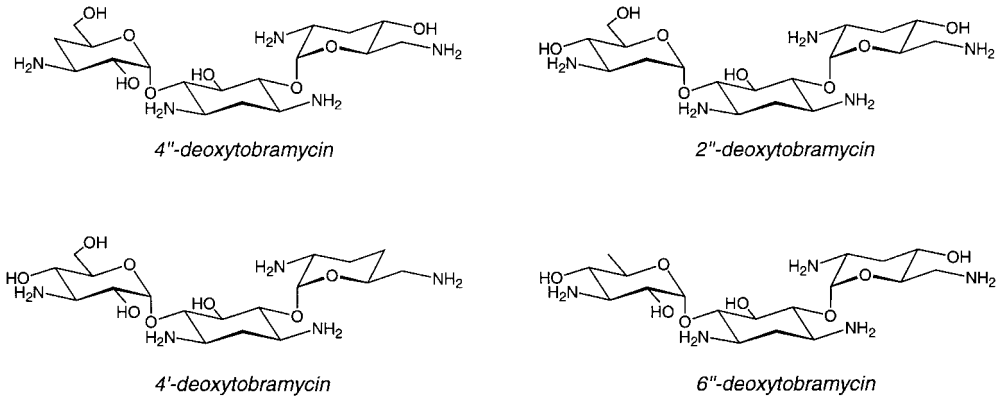

Figure 3. Deoxygenated tobramycin derivatives.[14]

are the deoxygenated derivatives lacking the 2′′-, 4′′-, and 4′-hydroxyls that are approximately five times more effective than the parent tobramycin in inhibiting the hammerhead ribozyme.[14] In contrast, removal of the primary 6′′-hydroxyl results in a poorer RNA binder. Our results indicate that when a hydroxyl group proximal to an amine is removed, higher inhibitory activity is observed. We attribute these observations to the increased basicity of an amino group when a neighboring hydroxyl group is removed.[14] Thus, the deoxygenated aminoglycoside derivatives may possess a higher positive charge density at a given pH when compared to their parent natural product. Our observations support the critical role of electrostatic interactions in RNA binding and suggest that altering the pK_a of amino groups is a possible mechanism for modulating the RNA affinity of synthetic ligands.

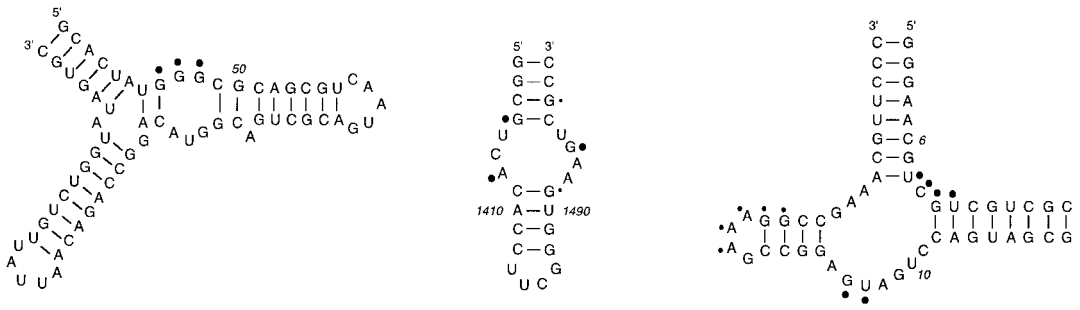

Figure 4. Proposed secondary structures for the RRE, ribosomal A-site model and the hammerhead ribozyme (HH16). Chemical footprinting experiments have shown that aminoglycosides protect the three consecutive G's within the RRE core,[18] as well as a discrete region of the asymmetric internal loop of the ribosomal RNA.[25] Footprinting and affinity cleavage studies have revealed two putative neomycin B binding sites on the hammerhead ribozyme.[28] The residues thought to be involved in aminoglycoside binding are marked.

Where do aminoglycosides bind? RNA is structurally and functionally sophisticated. It possesses a multitude of secondary structures that, together with numerous tertiary interactions, lead to complex folding patterns and the formation of potential binding pockets. The RNA molecules known to be targeted by aminoglycosides are seemingly unrelated. The lack of apparent sequence homologies points to the possibility of shape recognition rather than sequence recognition.

A concurring picture of RNA – aminoglycoside binding has been obtained by the identification of discrete binding regions in small RNA molecules using footprinting and NMR studies supported by computational techniques. In all cases, aminoglycoside antibiotics have been found to bind preferentially to single-stranded RNA regions containing loops and bulges.[18, 24–27] For example, chemical footprinting experiments have shown that aminoglycosides bind to a discrete domain within the RRE core element and protect the three consecutive G's (46 – 48) that are also essential for Rev binding (Figure 4).[18] Similarly, a discrete binding site was identified on the asymmetric internal loop of a model RNA mimicking the A site of *E. coli* 16S rRNA. Nucleotides strongly protected by paromomycin include G1405, A1408, and G1494 (Figure 4).[25] Preliminary footprinting and affinity cleavage experiments done in our laboratory have identified two putative neomycin B binding sites on the hammerhead ribozyme (Figure 4).[28] The residues found to be involved in binding are U13 and G14 on the enzyme and U8, C9, G10, U11 on the substrate. These residues are in close proximity in three dimensions, as observed in the crystal structure of a related hammerhead ribozyme, and are critical for the catalytic activity of the ribozyme. Molecular dynamics simulations support this view.[26] A secondary binding site including three consecutive A residues in a tetraloop has also been implicated (Figure 4).[28]

Why is the interruption of duplex RNA by a single-stranded region advantageous for binding small molecules? Weeks and Crothers have shown that the usually narrow and deep RNA major groove of a TAR RNA model is significantly widened in the region of a single base bulge and thus more accessible for a Tat-derived peptide ligand.[29] Most likely, aminoglycoside binding can be facilitated by similar recognition patterns. As an example, the NMR structures of the 16S rRNA A-site analogue complexed with neomycin-class aminoglycosides show that the aminoglycosides' binding site lies in the distorted extension of the major groove in the bubble region.[25] The NMR-based comparison of the 16S rRNA A site analogue in its free and paromomycin-bound structure demonstrates that a local conformational change takes place upon complex formation, thus stabilizing the rather flexible loop region.[30] Figure 5 illustrates intermolecular contacts in

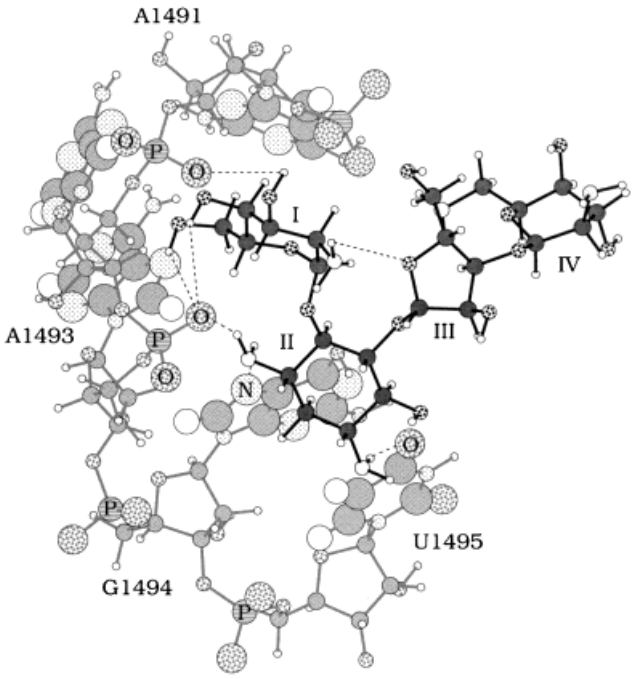

Figure 5. Partial NMR structure of a 16S rRNA A-site analogue (gray) complexed to paromomycin (black). Plausible contacts between rings I and II of paromomycin and the heterocycles of G1494 and U1495 as well as the 3′-phosphate of G1491 and the 5′-phosphate of A1493 are indicated by dashed lines. An intramolecular contact between rings I and II is also shown. The structure was solved by Puglisi and co-workers;[25] the coordinates were obtained from the Brookhaven Protein Data Bank (1pbr.pdb).

the complex.[25] In some cases of RNA – aminoglycoside complexes, the binding region is extended to a few stem base pairs in direct vicinity to looped or bulged regions.[25–27]

RNA – aminoglycoside binding is not limited to single-stranded RNA regions. Comparative thermal melting studies

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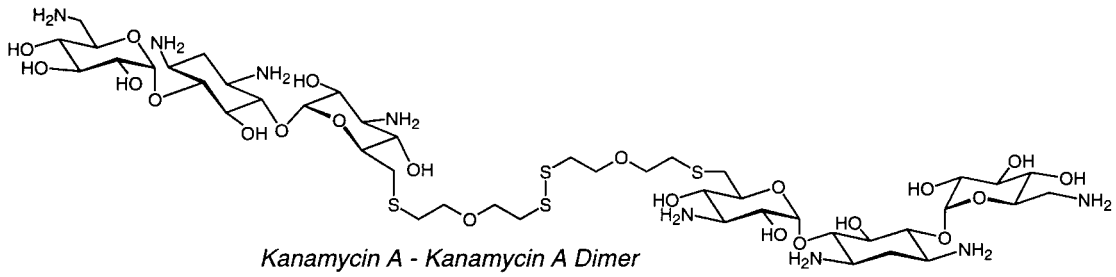
have shown that aminoglycosides can also bind to synthetic RNA duplexes that lack bulges (e.g., polyA · polyU).[31, 32] It is worth mentioning that no significant aminoglycoside binding is observed with the corresponding DNA duplex; this indicates the exceptional selectivity of aminoglycosides for RNA over DNA binding. Based on ^{19}F NMR studies, a recognition model proposing aminoglycoside binding in the deep RNA major groove has been suggested.[31] Binding of polycationic aminoglycosides in the major groove of A-form nucleic acids is not surprising. While in B-DNA the electrostatic potential distribution between the minor and major grooves is balanced, in A-DNA the major groove is associated with a much stronger negative potential.[33] Taken together, there are at present no indications for RNA minor-groove binding by aminoglycosides.

Do multiple binding sites for aminoglycosides exist? The antibiotics may bind to existing binding sites within the folded RNA or induce a conformational change, thus altering the three-dimensional structure of the host RNA. An important feature of both views is that several sites with different affinities may coexist in a given RNA molecule. To explore this possibility, we have designed and synthesized dimeric aminoglycosides (Figure 6).[34] These highly charged aminoglycoside derivatives show enhanced affinity to RNA when compared to their monomeric counterparts. In general, when two moderate or good RNA binders (e.g., tobramycin, neomycin B) are covalently linked, the ribozyme inhibitory activity of the derivative surpasses that of any natural aminoglycoside antibiotic. It might be expected that these molecules bind to RNA even more strongly than the amino-aminoglycosides, but surprisingly, this is not necessarily the case. The dimeric aminoglycosides are not stronger hammerhead inhibitors than 5″-amino-5″-deoxyneomycin B.[23,34] Seemingly, a certain saturation level is reached with regard to the number of amino groups and positive charges in a given ligand. Increasing the number of amino groups to eight or higher does not result in stronger binding to the hammerhead ribozyme. This further supports the notion of discrete binding sites, rather than simple electrostatic interactions.

The dimerization of aminoglycosides opens up an exciting avenue for the exploration of the existence of multiple binding sites within the tertiary structure of an RNA molecule. In principle, if two binding sites are in close proximity, a dimeric derivative can bind simultaneously to the two sites, resulting in stronger binding affinity. This concept was investigated by studying the inhibition of the *Tetrahymena* ribozyme L-21*Sca*I (388 nucleotides) by dimeric aminoglycosides. All dimeric aminoglycosides are substantially better inhibitors than any of the monomeric parent aminoglycosides.[35] As an example, the poor ribozyme binder kanamycin A ($IC_{50} = 9 \times 10^2$ μM) is converted to a strong ribozyme binder ($IC_{50} = 0.7$ μM) upon dimerization (Figure 6). The enhanced binding to the RNA is likely to be due to a higher number of amino groups in the dimeric derivatives that can be accommodated by larger or multiple binding sites on the *Tetrahymena* ribozyme.

How do aminoglycosides bind to RNA? Uhlenbeck and coworkers have shown that aminoglycoside antibiotics interact preferentially with the enzyme–substrate complex of the hammerhead ribozyme and inhibit the cleavage step.[13] Competition experiments between neomycin B and magnesium ions suggest that the dominating interactions between the hammerhead ribozyme and aminoglycosides are ionic in character, and that five magnesium ions are required to abolish inhibition of the ribozyme by neomycin B.[36] On the basis of pK_a values, five out of the six amino groups of neomycin B should be protonated at pH 7. It has therefore been suggested that five magnesium ions compete with a single neomycin B molecule for a binding site on the hammerhead ribozyme. Since the presence of these magnesium ions is critical for proper ribozyme function (including



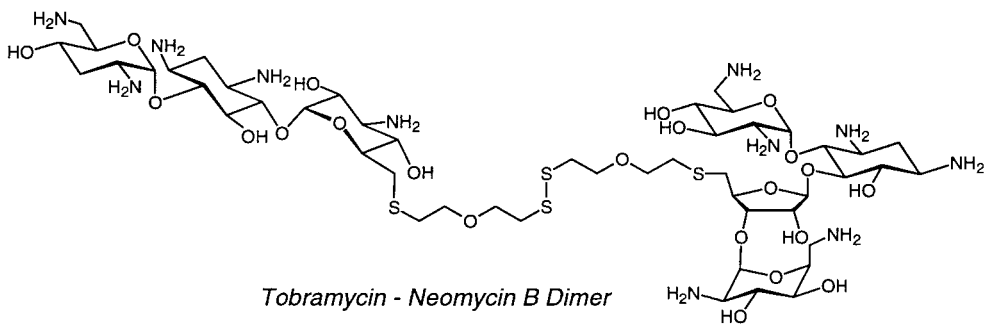


Figure 6. Examples of symmetric and nonsymmetric dimeric aminoglycoside derivatives.[34,35]

folding and catalysis), their replacement by neomycin B results in inhibition.

Our studies with deoxygenated tobramycin derivatives and amino-aminoglycosides strongly support the critical role of electrostatic interactions in RNA – aminoglycoside binding. The finding that 6″-amino-6″-deoxykanamycin A and kanamycin B have very similar ribozyme inhibitory activities led us to propose a hypothetical binding model.[23] Both derivatives have five amino groups, albeit in different positions of the aminoglycoside skeleton, and are likely to have a similar overall positive charge at a given pH. The stereochemical array of the positive charges of the two aminoglycosides can be superimposed after rotation and conformational changes. We have proposed that these aminoglycosides present a similar stereochemical array of positive charges. Our RNA – aminoglycoside recognition model emphasizes structural electrostatics: a specific, yet versatile and dynamic, three-dimensional projection of positively charged ammonium groups toward acceptor groups on the RNA binding site.[23]

Using molecular dynamics simulations of a crystallographically studied hammerhead ribozyme, Hermann and Westhof have recently uncovered a striking complementarity between the interionic Mg^{2+}—Mg^{2+} distances on the ribozyme and the intramolecular distances between the charged ammonium groups on aminoglycosides.[26] Docking experiments have demonstrated that numerous conformers of a number of aminoglycosides can place the ammonium groups at the sites normally occupied by the Mg^{2+} ions. It was suggested that the covalently linked array of ammonium groups is capable of displacing three to four magnesium ions and complements the negative electrostatic potential created by the RNA fold. This model suggests a three-dimensional electrostatic complementarity rather than highly specific contacts between the aminoglycoside and an RNA receptor site, and is in agreement with our experimental data and recent NMR studies.[24]

Beyond aminoglycoside antibiotics: While most of our discussion has been focused on aminoglycoside antibiotics as representative RNA binders, other small organic and inorganic molecules have been reported to interact with various RNAs. Most of these unique examples, including certain nonspecific binders, have been discussed by Chow and Bogdan.[2] In the light of the importance of electrostatic interactions in RNA binding and the proposed models discussed above, one may wonder about the possibility of utilizing other modes of binding for the construction of new RNA binders. In this section we address two questions that are of general interest.

Is there a precedent for RNA binders containing negatively charged residues? Wong and co-workers have synthesized aminoglycoside mimetics as RNA binders using a biased combinatorial approach.[37] A library consisting of neamine as a core and a variable dipeptide moiety was constructed by a multicomponent condensation. Each compound was individually tested for its ability to inhibit the Rev – RRE interaction. Nine out of 52 compounds tested were as good as or better inhibitors than neomycin B and were significantly better than neamine. Surprisingly, the best inhibitors contained two or three carboxyl groups (Figure 7). The RNA binding assay was performed at pH 7.2;[38] this results in a zwitterionic form of the ligands with an excess positive charge. Although these results remain unexplained, it is important to point out the novelty of RNA binders that contain negatively charged residues. A possible interpretation is that coordination of essential cations by the free carboxylate residues of the aminoglycoside mimetic may induce an RNA conformational change. Since these derivatives have not been tested for their binding to RNA alone, the possibility of a ternary complex formation (RRE RNA – aminoglycoside mimetics – Rev protein) cannot be excluded.

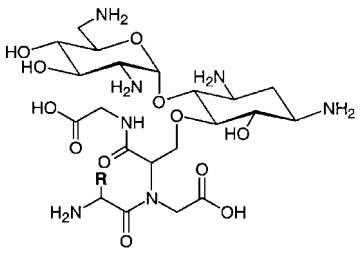


Figure 7. A general structure of the most active aminoglycoside mimetics synthesized by Wong and co-workers.[37]

Can different binding modes be combined to enhance RNA binding affinity? Wilson and co-workers have synthesized a series of nonglycosidic cationic small molecules and investigated their binding ability to defined RNA duplexes with and without bulge bases.[39] Complex stabilities were qualitatively estimated by comparing melting temperatures. Remarkable complex stabilization and selectivity for an RNA duplex containing a single bulged base over a normal RNA duplex have been obtained with a ligand consisting of a chloroacridine moiety covalently attached to 2,6-diaminopurine through an aminoalkyl linker (Figure 8). It is believed that the chloroacridine moiety intercalates into the RNA duplex and the 2,6-diaminopurine interacts with the bulged base.[39] The observed ΔT_m values and selectivity are very sensitive to the number of positive charges in the linker and the attached heterocycle. Not surprisingly, a positively charged linker is more effective than an uncharged alkyl linker. These examples illustrate that the combination of different binding modes within one ligand can lead to enhanced selectivity and binding affinity.

What does it take to make a good RNA binder? In general, low binding affinities and the lack of specificity are obstacles that have to be overcome in the development of new RNA-binding molecules. Due to the complexity of the problem and its relevance to the area of RNA – aminoglycoside interactions, we devote a short discussion to this important issue.

The strength of a given biomolecule – ligand complex can be rigorously determined by experimental measurement of binding (or dissociation) constants. Somewhat more subtle is the selectivity and specificity of binding. Selectivity is the preferential formation of one complex over another in a

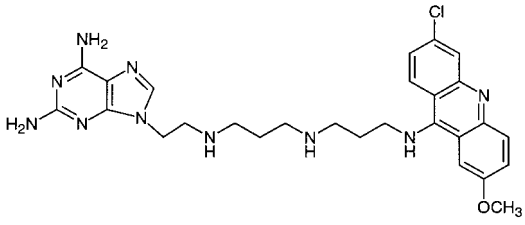


Figure 8. Diaminopurine-linked acridine synthesized by Wilson and co-workers.[39]

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ligand–biomolecule binding event. Specificity can be ascertained if a related ligand generates a different population of complexes. Specificity cannot be established, therefore, without proper comparison. Hence, specific binding implies selectivity, but selective binding is not necessarily specific.

A nucleic acid binder may be able to distinguish between DNA and RNA, thus showing selectivity for one type of nucleic acid over the other. For example, neomycin B does not significantly bind to DNA, but does bind to a number of RNA sequences. On the other hand, distamycin binds to certain DNA sequences only. Thus, neomycin B binds RNA specifically while distamycin is DNA-specific.

At the next level, an RNA binder might be able to distinguish between different RNA molecules within an RNA pool. For example, aptamers selected for lividomycin bind poorly to kanamycin A while aptamers selected for kanamycin A bind poorly to lividomycin.[40] Thus, lividomycin and kanamycin A bind specifically to their cognate aptamers. Similar observations have been reported for RNA aptamers selected for other aminoglycoside antibiotics.[27, 41] Another example is the specific binding of neomycin B and paromomycin as well as a number of synthetic aminoglycoside derivatives to an A-site analogue of the 16S ribosomal decoding region, as recently reported by Wong and co-workers.[42] Dissociation constants for the A-site analogue and a single-base mutated analogue were determined using surface plasmon resonance. Specific binding could be ascertained since different $K_{d(RNA\,I)}/K_{d(RNA\,II)}$ ratios were obtained. Fluorescence anisotropy measurements have been utilized by Rando and co-workers to study the binding specificity of aminoglycosides to RNA constructs derived from 16S rRNA and HIV-1 RRE.[43] Despite the limited specificity generally found in binding of aminoglycosides to natural RNA molecules, an inherent specificity of certain aminoglycosides to the 16S rRNA has been proposed.[43] Puglisi's NMR studies of 16S rRNA–aminoglycoside complexes provide insight into the elements of specificity at the atomic level (Figure 5). The neamine moiety of the aminoglycoside (rings I and II) is involved in well-defined interactions with specific heterocycles and phosphate groups of the RNA host, while the other two rings (III and IV) are spatially less confined. These rings form sequence-independent electrostatic contacts with the phosphodiester bonds of the RNA skeleton.[25]

In addition, a number of potential aminoglycoside binding sites may coexist on a single RNA molecule. An aminoglycoside may bind selectively to a certain site. If another aminoglycoside bound preferentially to a different site of the same RNA molecule, both aminoglycosides would bind site-specifically. Footprinting studies have revealed differential binding preferences of streptomycin and neomycin B for two distinct sites on 30S rRNA. Both aminoglycosides bind site-specifically to the 30S ribosomal subunit.[6]

In spite of these remarks, the issue of specificity is still not clearly resolved. It is widely believed that electrostatic interactions between a nucleic acid and its ligand are nonspecific in nature. An experiment often used to evaluate the contribution of electrostatic interactions to binding includes studying the binding interactions at increasing ionic strength. A decrease in binding affinity indicates a loss of electrostatic interactions and is commonly interpreted as an indication for nonspecific interactions. However, the distinction between specific and nonspecific RNA–aminoglycoside interactions based on studies dependent on ionic strength alone can contrast with the understanding of specificity as explained above. Hence, specific and electrostatic binding are not mutually exclusive.

Despite our progress in understanding RNA–small molecule interactions, the design of a suitable ligand for a vaguely known RNA receptor is still challenging and empirical in nature. General recognition rules derived from studying RNA–aminoglycoside interactions have been partially elucidated and are summarized below.

The overall charge of a ligand appears to be critical for high binding affinity. Aminoglycosides that bind RNA most efficiently have at least six amino groups (neomycin B, 6″-deoxy-6″-aminotobramycin, 6″-deoxy-6″-aminoneomycin B, dimeric aminoglycosides, etc.). However, increasing the number of amino groups does not lead to increasing binding affinities in a small RNA molecule such as the hammerhead ribozyme. Larger RNA molecules that have elaborate secondary and tertiary structural elements may provide larger or multiple binding sites. Increasing the size and charge of the recognition domain may therefore become beneficial when targeting large RNA molecules.

Designing an organic molecule by incorporating a certain number of amino groups may be a naive approach. It is important to consider the basicity of every individual basic group and the intramolecular modulation of their pK_a values in a given structure. SAR investigations of deoxygenated aminoglycosides demonstrated how the presence of hydroxyl groups can influence binding affinity.[14] Although hydroxyl groups may be involved in favorable intermolecular hydrogen bonds, placing them in close proximity to primary amino groups might significantly decrease the pK_a values of the ammonium groups. Therefore, if hydroxyl groups are to be incorporated into a designed RNA binder, their position has to be carefully engineered.

Another significant factor for the design of an RNA binder is the choice of the molecular scaffolding that governs the display of the recognition elements. Efficient RNA binding can be achieved by strategic incorporation of ammonium groups with predetermined three-dimensional projection. Linear polyamines, such as spermine and spermidine, are inferior RNA binders when compared to aminoglycoside antibiotics. In general, their binding affinities are lower and they are believed to bind nonspecifically to RNA. The linear structures may be too flexible and the amino groups may be in unfavorable proximity. Consequently, the ammonium groups may not be able to simultaneously satisfy the electrostatic complementarity on negatively charged surfaces within the three-dimensional structure of RNA receptors.

In aminoglycoside antibiotics, the ammonium groups are located on relatively rigid six-membered rings that can adopt various orientations with respect to one another. The combination of fragment rigidity and inter-ring conformational flexibility may be the key to the high affinity of aminoglycosides to RNA. Indeed, molecular dynamics simulations suggest that aminoglycoside antibiotics can bind to the

hammerhead ribozyme by adopting numerous conformations and various projections of their charged ammonium residues.[23, 26] These positive charges are well suited to meet the required charge complementarity to the RNA binding site. Thus we propose a flexible and versatile induced-fit model for binding aminoglycoside antibiotics to RNA molecules.

With our increasing understanding of RNA tertiary structure, a promising approach that could lead to higher binding affinities and selectivity is the creation of a ligand consisting of two different moieties attached by a linker. The molecule can be a dimeric form of a known binder or can consist of two distinct moieties that bind to RNA in different modes (e.g., groove binding and intercalation). In both cases the length and nature of the linker is critical. Higher binding affinities can be expected owing to a favorable entropic factor compared with the binding of the two monomeric counterparts. Slower *off* rates and faster *on* rates can result from one of the two moieties being permanently bound to the RNA molecule.

Summary and Implications

RNA molecules play key roles in essential biological processes, such as protein synthesis, transcriptional regulation, splicing and retroviral replication. The structural diversity of RNA and the lack of known RNA repair mechanisms make RNA a challenging, yet very important, target for therapeutic intervention. In particular, the increasing problem of bacterial resistance to antibiotics as well as the need for advanced antiretroviral agents make the search for novel RNA binders of crucial importance.

Our understanding of the modes in which RNA is recognized by other ligands is far from being comprehensive. It remains to be seen whether the various approaches discussed above will materialize into general RNA binding motifs. As it appears now, the design of new RNA binders is likely to progress hand in hand with the advances in our general knowledge of RNA structure, folding and catalysis, as well as RNA–protein interactions. Until we develop a deeper understanding of these key features, the design of new RNA binders is likely to remain an empirical task. This is clearly an intriguing and stimulating challenge!

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