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Aminoglycoside Binding to Human and Bacterial A-Site rRNA Decoding Region Constructs☆

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Abstract—The 16S bacterial ribosomal A-site decoding rRNA region is thought to be the pharmacological target for the aminoglycoside antibiotics. The clinical utility of aminoglycosides could possibly depend on the preferential binding of these drugs to the prokaryotic A-site versus the corresponding A-site from eukaryotes. However, quantitative aminoglycoside binding experiments reported here on prokaryotic and eukaryotic A-site RNA constructs show that there is little in the way of differential binding affinities of aminoglycosides for the two targets. The largest difference in affinity is 4-fold in the case of neomycin, with the prokaryotic A-site construct exhibiting the higher binding affinity. Mutational studies revealed that decoding region constructs retaining elements of non-Watson–Crick (WC) base pairing, specifically bound aminoglycosides with affinities in the μM range. These studies are consistent with the idea that aminoglycoside antibiotics can specifically bind to RNA molecules as long as the latter have non-A form structural elements allowing access of aminoglycosides to the narrow major groove. © 2001 Published by Elsevier Science Ltd.

Introduction

Aminoglycoside antibiotics are aminocyclotol-containing molecules of the structural type shown in Scheme 1. These molecules have a general capacity to bind to nucleic acids, often by means of electrostatic interactions between the phosphate backbone of the nucleic acids and the positively charged amino groups of the aminoglycosides.[1,2] Aminoglycoside antibiotics are clinically used to combat Gram-negative bacterial infections because they are able to interfere with protein translation in prokaryotes as a consequence of binding to the A-site decoding region of rRNA.[3,6] As a consequence of binding to this region, the aminoglycosides cause mistranslation of mRNA as well as causing premature termination of message readout.[4,7] This binding to the prokaryotic rRNA cannot be entirely specific inasmuch as aminoglycosides can also cause mistranslation in eukaryotes, as is clear from the toxicity that results from the clinical use of these drugs.[8] In fact, this lack of specificity is exploited in the imaginative use of aminoglycosides to suppress nonsense mutations in the cystic fibrosis transmembrane conductance regulator in a proposed treatment of cystic fibrosis[9] and in the restoration of dystrophin function in muscular dystrophy.[10] An important question that emerges from these kinds of observations is how specific are the aminoglycosides with respect to decoding region RNA structure, and what general features of RNA molecules are recognized?

It is already clear that many different kinds of RNA molecules are targets for aminoglycoside binding. In addition to the A-site decoding region, aminoglycosides are also known to bind to HIV-RRE RNA,[11,12] HIV-TAR RNA,[13] thymidylate synthase mRNA,[14] a hammerhead ribozyme,[15] as well as large numbers of RNA molecules (aptamers) selected to bind to aminoglycosides.[16–18] All of these structures have certain structural features in common. Most notably, they all contain non-duplex regions of RNA. That is, the aminoglycosides appear to prefer to bind to structures frequently containing internal bulges and bubbles. This is likely because the major groove in A-form duplex RNA is too narrow for the aminoglycoside to fit sterically.[19] Interestingly, though, aminoglycosides may also specifically bind to regions of RNA containing only a non-canonical base pair, but otherwise without bubble or bulge regions.[20] This suggests that many distinct domains with structural perturbations in RNA molecules can provide a binding site for aminoglycoside

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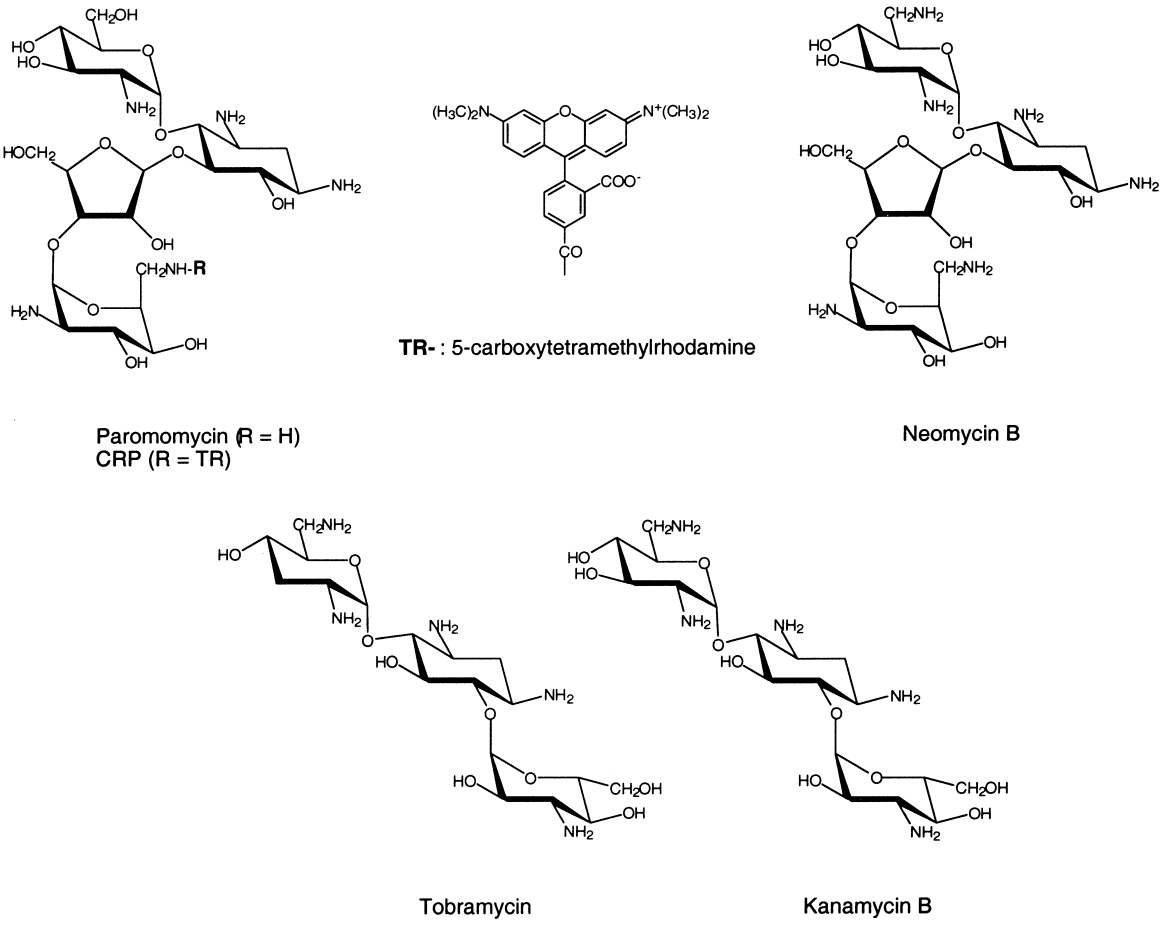
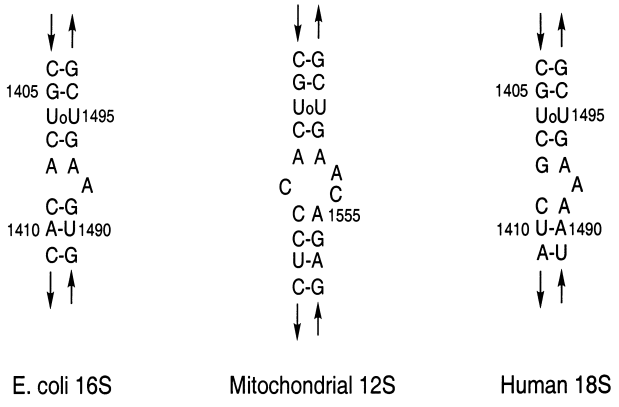


Scheme 1. Structures of aminoglycosides used in studies.

molecules. While it is clear that the bulged prokaryotic A-site decoding region, shown in Scheme 2 along with its 12S mitochondrial and 18S human counterparts, is the physiologically relevant binding site for aminoglycosides,[21,22,23] quantitative determinations on the specificity of aminoglycoside binding to this region are lacking.

The possibility of approaching this problem is made feasible by the fact that truncated A-site rRNA constructs can be prepared which exhibit biochemical properties, including aminoglycoside binding, reminiscent of intact 16S rRNA containing ribosomes.[21,22,24]



Scheme 2. Prokaryotic and eukaryotic A-site decoding regions.

That these analogues can be taken as relevant models for the analysis of the structure and molecular function of full-length RNA molecules is exceedingly important. Strong arguments for the cogency of this viewpoint are to be found in NMR and X-ray structural studies on small RNA analogues of the bacterial A-site decoding region[21] and HIV RRE and TAR regions.[25–30] In addition to having some confidence in the notion that mini-RNA constructs are functional analogues of their full-length cognates it is also important to be able to quantitatively measure the interactions between ligands and RNA molecules. Quantitative fluorescence methods have been developed which allow for the accurate measurement of binding constants between aminoglycosides and RNA receptors,[17] thus addressing the issue of binding specificity directly.

Fluorescent aminoglycosides, of the type shown in Scheme 1 [5-Carboxytetramethylrhodamine-labeled paromomycin (CRP)], readily bind to RNA receptors and this binding is easily followed by fluorescence polarization or quenching methods.[16,22,31] Dissociation constants for unmodified aminoglycosides are determined in a straightforward manner in competition assays.[22,31] Using these assay methods, it could be readily shown that aminoglycosides bind to prokaryotic A-site rRNA constructs with K_D values in the 0.1–10 μM range.[22] A question that emerges relates to whether there are any special features of the prokaryotic A-

site construct which are essential for specific aminoglycoside binding. Results from chemical modification studies suggest that various A-site mutants may have substantially reduced abilities to bind aminoglycosides, and that there might be significant specificity involved in the binding of aminoglycoside to the A-site region[23,32,33] not found on studies with other RNA targets for aminoglycosides.

An important aspect of any study on decoding region constructs relates to a comparison of the human and bacterial A-site variants with respect to aminoglycoside binding affinities. This is because it might be supposed that important differences in affinities may be found which could at least partially explain the selective toxicity of aminoglycosides for prokaryotes. Moreover, these studies further address the issue of the inherent specificity of aminoglycoside binding to important pharmacological targets. In fact, as demonstrated here, there are only small differences in binding affinities observed for aminoglycosides for the human cytoplasmic and bacterial A-site decoding regions. This suggests that the selective toxicity of aminoglycosides for prokaryotes is only partially explained by differential binding affinities to the RNA sites themselves and the selective toxicity may be more importantly explained by phenomena such as selective uptake into prokaryotes. In addition, mutations in the decoding region constructs have, in general, relatively minor quantitative effects on aminoglycoside binding.

Materials and Methods

Materials

Neomycin sulfate, paromomycin sulfate, kanamycin B sulfate and tobramycin sulfate salt were purchased from Sigma Inc. and were used without further purification. 5-Carboxytetramethylrhodamine succinimidyl ester was purchased from Molecular Probes Inc. CRP was prepared as previously reported.[22] Oligonucleotides were systhesized by Integrated DNA Technologies, Inc. RNA transcripts were produced using RiboMax large-scale RNA production kit from Promega Inc. Nick columns from Pharmacia Inc. were used for RNA purification.

Methods

RNA synthesis and purification. Transcripts of the A-site decoding region of rRNA and its variants were prepared in vitro with a RiboMax RNA production kit using synthetic oligonucleotide templates according to the procedures provided by the manufacturer. Pure RNA transcripts were made by separating them from salts and nucleotides in the reaction mixture using Nick columns. Secondary structures for the RNA constructs were predicted using the Mfold program.[34]

Fluorescence measurements

CRP concentrations were determined spectroscopically at 550 nm using a molar extinction coefficient of $6.00\times10^4\ M^{-1}\ cm^{-1}$. Fluorescence anisotropy measurements were performed on a Perkin-Elmer LS-50B luminescence spectrometer equipped with a thermostat accurate to $\pm0.1\ ^\circ C$. The tracer solution was excited at 550 nm and monitored at 580 nm. The integration time was 5 s. For every point, six measurements were taken, and their average values were used for calculation. Measurements were carried out in a buffer solution containing 150 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 20 mM HEPES (pH 7.5). Prior to measurements, RNA transcripts were renatured by incubating in binding buffer [150 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 20 mM HEPES (pH 7.5)] for 3 min at 90 °C followed by slow cooling to 25 °C.

Determination of dissociation constants

Eq (1) was used for the determination of the dissociation constant for the interactions between RNA and CRP (K_d).

$$A = A_0 + \{\Delta A[RNA]_0 + [CRP]_0 + K_d - [([RNA]_0 + [CRP]_0 + K_d)^2 - 4[RNA]_0[CRP]_0]^{1/2}\}/2^1$$

where A and A_0 are the fluorescence anisotropy of CRP in the presence and absence of RNA, respectively, and ΔA is the difference between the fluorescence anisotropy of CRP in the presence of an extrapolated infinite concentration of RNA minus the fluorescence anisotropy in the absence of RNA. $[RNA]_0$ and $[CRP]_0$ are the initial concentrations of RNA and CRP, respectively.

Eq (2) is used for the calculation of the K_D values in the competition binding assay.

$$[\text{aminoglycoside}]_0 = [K_D(A_\infty - A)/K_d(A - A_0) + 1] \times \left[[RNA]_0 - K_d(A - A_0)/(A_\infty - A) - [CRP]_0(A - A_0)/(A_\infty - A)\right]^2$$

where K_D is the dissociation constant between the RNA and the aminoglycosides. $[\text{aminoglycoside}]_0$ is the initial concentration of the aminoglycosides. Both K_d and K_D were determined by non-linear curve fitting using the equations described above[16,22] and are presented as a mean value of three independent measurements.

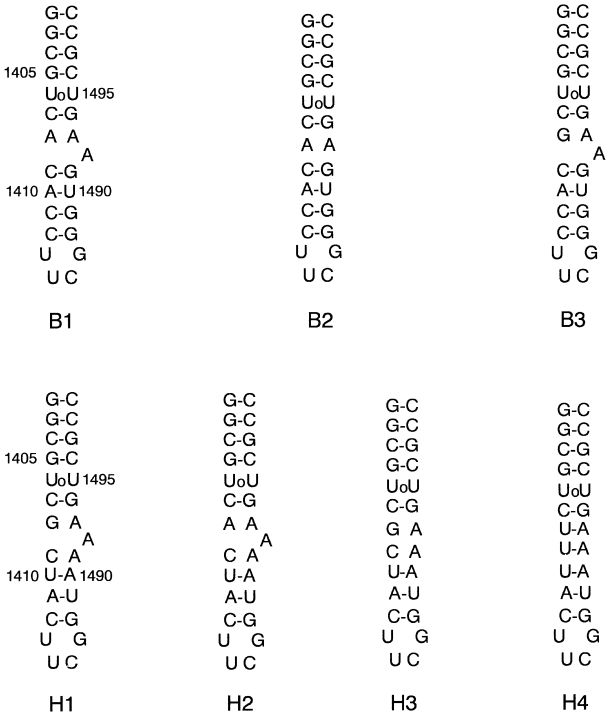
Results

The binding of aminoglycosides to the prokaryotic and human A-site constructs

While several prokaryotic A-site rRNA constructs have been designed and studied,[24,31–33] the very simple construct B1 shown in Scheme 3, whose structure has been examined by NMR,[21] was chosen for study here as the prokaryotic A-site construct. Its cognate human cytoplasmic A-site construct H1 was also prepared, and is

shown in Scheme 3 as well. As shown in Figure 1(A), the B1 construct binds the fluorescent paromomycin analogue CRP (Scheme 1) specifically and in a saturable fashion with a $K_d = 0.20\,\mu M$. Binding to the analogous 18S human A-site construct H1 (Scheme 3) is shown in Figure 1(B), and provides a $K_d = 1.36\,\mu M$. Aminoglycoside binding to the eukaryotic A-site construct is not an unexpected result because aminoglycosides are thought to bind to the 18S human A-site.[8–10]

In subsequent experiments, dissociation constants were determined for the unmodified aminoglycosides by competition experiments with the fluorescent probe CRP. When competition experiments were performed with the aminoglycosides neomycin, paromomycin, tobramycin and kanamycin (Scheme 1), the results shown in Table 1 were obtained. In Figure 2 (A–D) binding isotherms are shown for the competition experiments with the prokaryotic 16S A-site construct and in Figure 3 (A–D) binding isotherms are shown for the human 18S A-site construct. What is extremely noteworthy, and perhaps somewhat surprising, is the degree to which the binding affinities are so similar. The largest differential in binding is found with neomycin, and the binding affinity difference here is less than 5-fold. The dissociation constants for paromomycin, kanamycin, and tobramycin are within experimental error of each other. Thus, the only differential in binding affinity is observed with neomycin.



Scheme 3. Decoding region A-site constructs studied.

The binding of aminoglycosides to mutant prokaryotic A-site constructs

These experiments were performed in order to probe the relationship between the prokaryotic A-site RNA structure and the binding of aminoglycosides. Previous studies clearly demonstrate the need for non-WC duplex elements for the specific binding of aminoglycosides to RNA molecules.[12,14] In the case of the prokaryotic construct under investigation here, aminoglycoside binding clearly involves the pocket formed by the bulged A1492 residue.[21] The importance of A1492 residue for aminoglycoside binding was probed with the two structures B2 and B3 shown in Scheme 3. Construct B2 (A1492Δ) is unable to bind to aminoglycosides (Table 2), confirming the general view that generally bulged and/or bubble structures are important for aminoglycoside binding. It has been pointed out that an important difference between prokaryotic and eukaryotic A-site regions with respect to aminoglycoside binding is the substitution of a G for A at position 1408 of the prokaryotic construct.[35] The A1408G construct was prepared B3 (Scheme 3) and tested for aminoglycoside

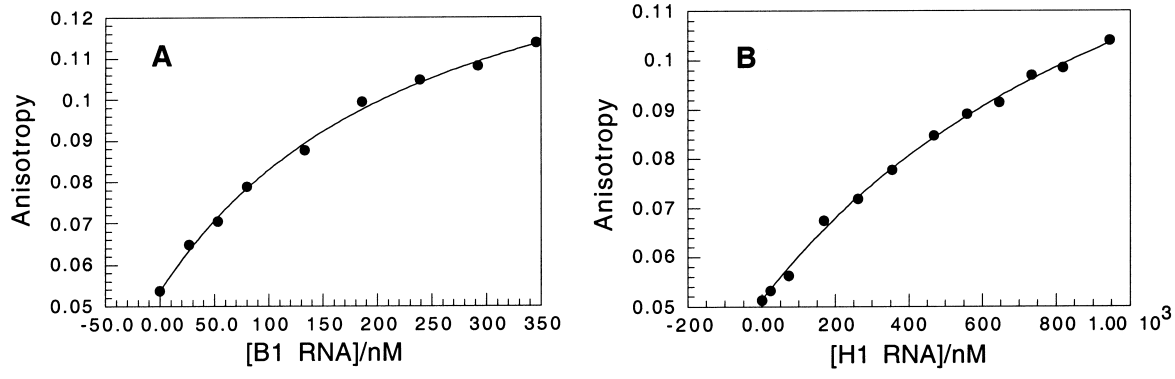


Figure 1. Fluorescence anisotropy of fluorescently labeled paromomycin (CRP) (20 nM) as a function of 16S rRNA construct, B1 (A) and 18S rRNA construct, H1 (B) concentrations. In these experiemtnts, increasing concentrations of the RNA constructs were added to 20 nM CRP under the conditions described in Materials and Methods.

Table 1. Dissociation constants for binding of aminoglycosides to A-site constructs (K_d (binding to CRP), K_D (binding to aminoglycosides), (μM))

RNA	CRP (K_d)	Neomycin	Paromomycin	Kanamycin	Tobramycin
B1	0.20±0.042	0.053±0.0035	1.65±0.35	1.25±0.07	1.40±0.24
H1	1.36±0.18	0.26±0.036	2.20±0.17	1.37±0.21	1.57±0.21

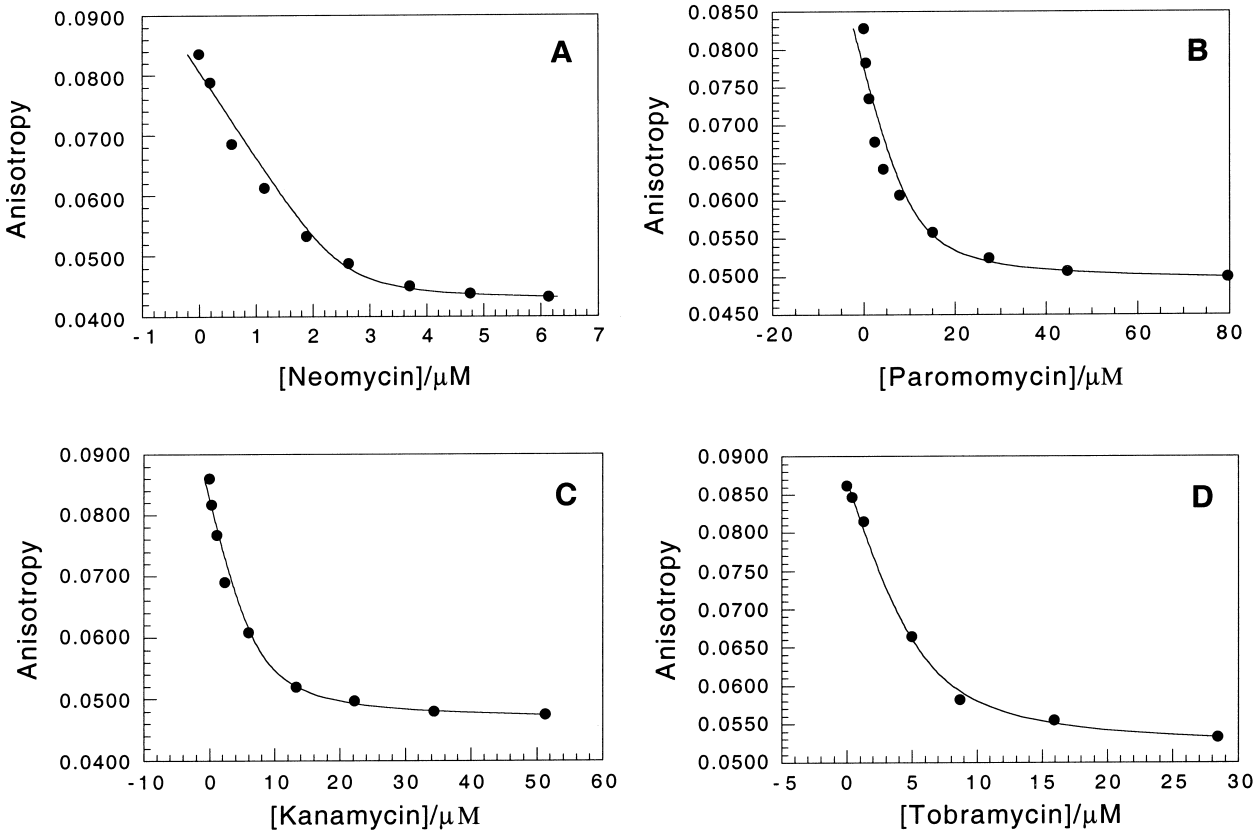


Fig 2. A-D. Fluorescent anisotropy of fluorescently labeled paromomycin (CRP) (20 nM) containing 16S rRNA construct, B1 as a function of neomycin (A), paromomycin (B), kanamycin (C) and tobramycin (D) concentrations.

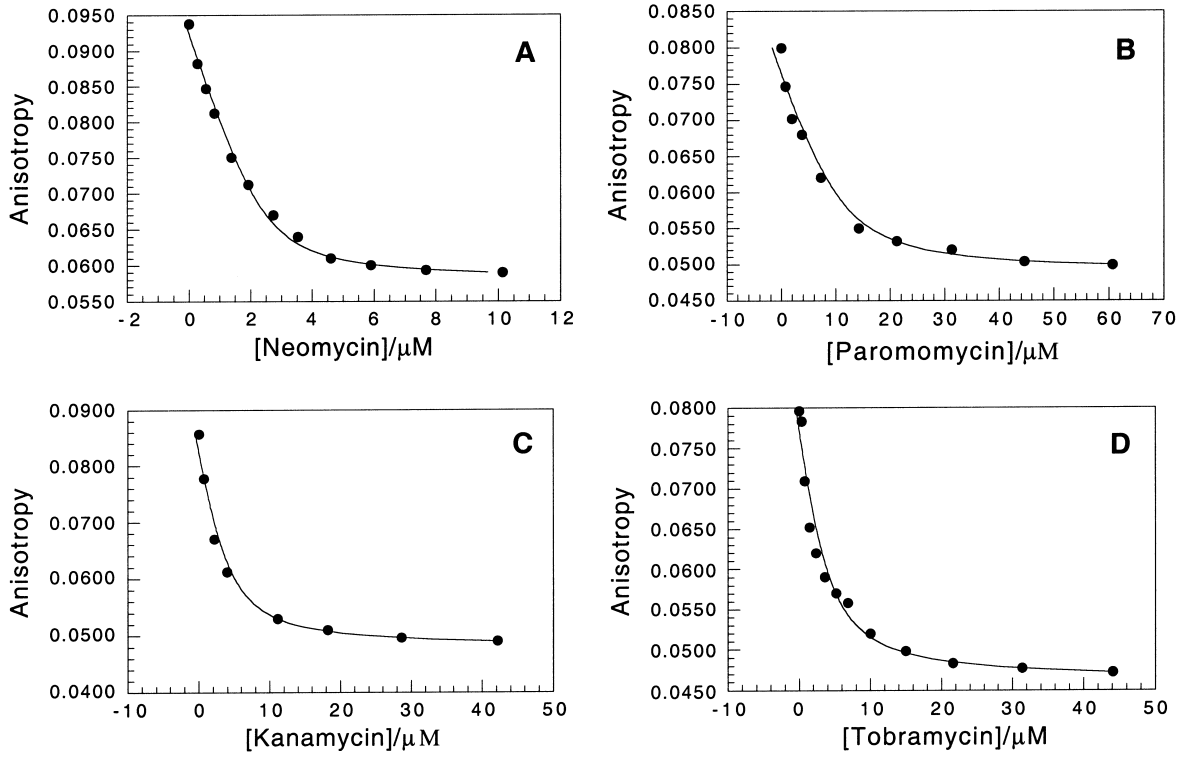


Fig 3. A-D. Fluorescence anisotropy of fluorescently labeled paromomycin (CRP) (20 nM) containing 18S rRNA construct, H1 as a function of neomycin (A), paromomycin (B), kanamycin (C) and tobramycin (D) concentrations.

Table 2. Dissociation constants for binding of aminoglycosides to mutant prokaryotic A-site constructs (K_d, K_D (μM))

RNA	CRP (K_d)	Neomycin	Paromomycin	Kanamycin	Tobramycin
B1 (WT)	0.20±0.042	0.053±0.0035	1.65±0.35	1.25±0.07	1.40±0.24
B2	NB[a]				
B3	0.90±0.11	1.1± 0.076	2.0±0.078	6.0±0.085	7.5±0.47

[a]No binding.

Table 3. Dissociation constants for binding of aminoglycosides to mutant eukaryotic A-site constructs (K_d, K_D (μM))

RNA	CRP (K_d)	Neomycin	Paromomycin	Kanamycin	Tobramycin
H1(WT)	1.36±0.18	0.26±0.036	2.20±0.17	1.37±0.21	1.57±0.21
H2	0.60±0.12	0.093±0.025	3.61±0.43	0.64±0.02	1.39±0.25
H3	0.52±0.17	0.058±0.012	2.05±0.071	1.14±0.19	0.81±0.071
H4	NB[a]				

[a]No binding.

binding, and as shown in Table 2, generally diminished affinities for aminoglycosides are in evidence. The most pronounced difference in binding is found with neomycin with an approximately 20-fold lessened affinity in the case of the mutant. Therefore, the prokaryotic A-site construct behaves in a fashion consistent with expectations with respect to aminoglycoside binding.

The binding of aminoglycosides to mutant eukaryotic A-site constructs

A series of eukaryotic constructs were also prepared (Scheme 3) and studied with respect to their abilities to bind aminoglycosides. First, the G1408A mutant H2 was prepared, and its affinities for aminoglycosides were determined (Table 3). This mutation is in essence the converse of the A1408G construct in the prokaryotic series B3. As can be seen in Table 3, enhanced neomycin binding is observed here, and the result is similar to that found with B3.

Further mutations in the eukaryotic series were studied to probe the relationship between RNA structure and aminoglycoside binding. Interestingly, deletion of the bulged A (A1492Δ) H3 had little affect on aminoglycoside binding in the eukaryotic series as shown in Table 3. This may be because the base pairs flanking this bulged A are themselves non-canonical. In fact, the G1408U + C1409U + 1492Δ mutant H4 (Scheme 3) again showed no binding of aminoglycosides.

Discussion

The general issue of the possible design of selective antagonists to particular RNA structures is of considerable interest. A variety of natural products, often antibiotics, interact with RNA molecules, and it is reasonable to address the issue of binding specificity with at least some of these molecules.[4,8] Among the antibiotics that interact with RNA, aminoglycosides are perhaps the most thoroughly studied.[8] Aminoglycosides have been found to bind to a large number of RNA molecules with dissociation constants in the low μM range. These RNA molecules include rRNA decoding region constructs,[21–23] HIV RRE and TAR RNA constructs,[11–13] a hammerhead ribozyme,[15] thymidylate synthase mRNA,[14] and a variety of RNA molecules (aptamers) selected from randomized pools to bind to aminoglycosides.[16–18] As a caveat though, it should also be noted that it has not always been directly demonstrated that specific aminoglycoside binding occurs in all of these enumerated instances. By specific binding, we refer to saturable and stoichiometric binding. Given the tendency of aminoglycosides to bind to nucleic acids through electrostatic interactions, specific binding is not always easy to discern.

An important question to address is whether a clear-cut relationship exists between local RNA structure and the ability of the RNA molecules to specifically bind aminoglycosides. That is, whether aminoglycosides prefer to bind to certain types of secondary structural units of RNA. A definitive answer to this question cannot be provided at present, although as a rule, it is clear that aminoglycosides only effectively bind to RNA molecules that have internal bubbles, bulges and other non-Watson–Crick (WC) duplex elements.[12,14,20] Presumably, the narrow A-site major groove in the WC-duplex is too narrow to allow effective access of aminoglycosides to the bases.[19] Without interactions with the bases, aminoglycoside-RNA recognition is only mediated by electrostatic interactions between the positively charged aminoglycoside and the phosphate backbone of the RNA molecules.[1,2] This typically leads to relatively weak, non-stoichiometric and, hence, non-specific, interactions between the aminoglycoside and the RNA molecule.

Given the wide spread binding of aminoglycosides for a variety of RNA structures, it is of substantial interest to question whether aminoglycosides exhibit substantial specificity with respect to their interactions with their pharmacological target; the 16S rRNA A-site decoding region of prokaryotes.[5] It is already clear that randomization and reselection of a 16S rRNA A-site decoding region construct against the aminoglycoside neomycin leads to a manifold of binding solutions.[36] This observation would be consistent with what is generally

understood concerning general lack of observed high specificity in the interactions of aminoglycosides with RNA molecules. On the other hand, NMR investigations on the binding of aminoglycosides to a truncated A-site construct suggest a substantial element of specificity in the binding.[21] In the current study, a highly reliable and quantitative fluorescence assay is used to approach the issue of aminoglycoside binding specificity for human cytoplasmic 18S and bacterial 16S A-site decoding region constructs.

On physiological grounds there is the known selective toxicity of the aminoglycoside antibiotics for bacteria to account for.[4] This fact does not immediately imply that aminoglycosides only bind weakly to eukaryotic targets. Selective toxicity could be attributed to several causes, only one of which might be related to a substantially decreased affinity of aminoglycosides for eukaryotic decoding regions versus their prokaryotic counterparts. Substantial evidence exists implicating selective permeation mechanisms for the uptake of the positively charged aminoglycosides into bacteria.[37–40] The drugs appear to be transported actively and concentrated in bacteria.[41] Finally, it is also possible that functional protein translation on bacterial ribosomes is particularly sensitive to aminoglycoside binding, or that mistranslated proteins are particularly lethal to the bacteria.

The simplest possibility of these possibilities to test relates, of course, to binding affinities. To test this notion, two rRNA A-site decoding region constructs were studied. The prokaryotic construct is exactly the same as the construct used in the NMR studies,[21] and eukaryotic construct is its analogue. The two A-site constructs are significantly different in their structures (Scheme 2). While both constructs contain a single A at an asymmetric bulge, the local environment surrounding the bulge is different. In the case of the mammalian construct, the A bulge is flanked by GA and CA non-canonical base pairs. On the other hand, the prokaryotic A-site contains a flanking AA non-canonical base pair and a WC CG base pair.

Perhaps surprisingly, the two constructs showed very similar binding affinities for aminoglycosides. The greatest difference in binding affinities proved to be approximately 4-fold, and that was for neomycin. The measured binding constants for paromomycin, kanamycin, and tobramycin were virtually identical, although interestingly substantial differences in affinities (approx. 7-fold) were found with CRP, the fluorescent paromomycin derivative. These results are consistent with the general patterns of aminoglycoside binding found with several different kinds of RNA constructs in which aminoglycoside binding is found with dissociation constants generally in the μM range. As stated above, as long as non-WC type elements are found, aminoglycoside binding is observed. In the case of the A-site constructs, the asymmetric A bulges are conspicuous non-WC structural elements.

Removal of the A bulge in the prokaryotic construct B2 led to an abolition of aminoglycoside binding as measured with CRP even though other non-canonical elements are still present. Interestingly though, the A1408G mutant B3 still bound aminoglycosides, although the affinity for neomycin was reduced by approximately 20-fold compared to wild type (WT) B1. A1408 is a highly conserved site in prokaryotes, and the substitution of a G at this site produces aminoglycoside resistance.[42] The equivalent position in the eukaryotic A-site construct also contains a G.

The G1408A mutant H2 was prepared and studied with respect to aminoglycoside binding. This mutation is analogous to the A1408G construct in the prokaryotic series B3. Enhanced neomycin binding is observed here, a result in line with results from studies on B3. Deletion mutations in the eukaryotic series were also studied. Deletion of the bulged A (A1492Δ) H3 had little effect on aminoglycoside binding in this series. This result runs counter to the one found in the bacterial series, and may be due to the fact that the base pairs flanking this bulged A are themselves non-canonical in the eukaryotic case. Consistent with this notion is the observation that the G1408U + C1409U +1492Δ mutant H4 again did not bind aminoglycosides.

It is clear that overall the quantitative aminoglycoside binding studies reported here did not show enormous differences in binding affinities between the human 18S decoding region construct and its bacterial 16S counterpart. The most significant difference is observed with neomycin, and this is only approximately 4-fold. Inasmuch as the small decoding region constructs used here and elsewhere are approximate functional surrogates for full length rRNA, then the selective toxicity of aminoglycosides for bacteria may be more related to pharmacokinetic issues rather than inherent binding affinities. However, it is also quite clear that whatever the in vivo differences in binding are for aminoglycosides they are not so large that aminoglycosides are non-toxic for human use.[8]

The data should not be construed to suggest that there is little or no specificity observed among the RNA constructs. As mentioned above, A1408G (B3) in the bacterial construct showed decreased binding affinities for the aminoglycosides compared to WT, especially in the case of neomycin, where an approximately twenty-fold decrease in affinity is measured. This result is in accord with published in vivo data on bacterial sensitivities[35] and suggests that the truncated decoding region analogues studied are representative of their full-length counterparts. A1408G confers resistance to a subclass of aminoglycosides, such as neomycin, which contain an amino group at the 6′ position of ring 1.[35] Minimum inhibitory concentrations for neomycin and paromomycin, which is devoid of the 6′ amino group, are 5.3 and 5.9 μM respectively for WT bacteria. In the case of the A1408G mutant, the values are 680 and 24 μM, respectively.[35] These data parallel the binding observations reported here on the A1408G mutant (B3), where neomycin affinity is reduced approximately 20-fold, while paromomycin binding is similar to WT.

In conclusion, the 16S prokaryotic A-site decoding region construct and its 18S human cognate construct can specifically bind aminoglycosides, mostly in the low μM range. The affinity for this interaction is typical of many of RNA aminoglycoside binding domains.[13,14,22,23] Specific binding does require non-WC duplex regions however, but otherwise specific binding affinities are only moderately affected by structural alterations. The effects of structural modifications in the RNA constructs on neomycin binding is greater than for the other aminoglycosides tested. However, overall there do not appear to be discrete structural switch elements that by themselves prevent aminoglycoside binding, as long as non-WC regions are present in the decoding region constructs. In other words, there does not appear to be any special specificity involved in the binding of aminoglycosides to the decoding regions. Having said this it will also be of interest to determine whether the same aminoglycoside-decoding region binding patterns are also manifest in intact 30S prokaryotic ribosomal subunits. Recently, the structure of 30S subunit was determined[43] and insights into the manner in which antibiotics bind to this subunit have been provided.[44] The structure of the A-site decoding region appears to be similar, but certainly not identical, to the prokaryotic decoding region construct whose structure was determined by NMR analysis[21] and is under consideration here. It will be of interest to analyze the binding of the various aminoglycosides to both eukaryotic and prokaryotic ribosomes to determine if the specificity of binding is different than observed here with the RNA constructs.

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