Specificity of aminoglycoside antibiotics for the A-site of the decoding region of ribosomal RNA

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Background: Aminoglycoside antibiotics bind to the A-site of the decoding region of 16S RNA in the bacterial ribosome, an interaction that is probably responsible for their activity. A detailed study of the specificity of aminoglycoside binding to A-site RNA would improve our understanding of their mechanism of antibiotic activity.

Results: We have studied the binding specificity of several aminoglycosides with model RNA sequences derived from the 16S ribosomal A-site using surface plasmon resonance. The 4,5-linked (neomycin) class of aminoglycosides showed specificity for wild-type A-site sequences, but the 4,6-linked class (kanamycins and gentamicins), generally showed poor specificity for the same sequences. Methylation of a cytidine in the target RNA, as found in the *Escherichia coli* ribosome, had negligible effects on aminoglycoside binding.

Conclusions: Although both 4,5- and 4,6-linked aminoglycosides target the same ribosomal site, they appear to bind and effect antibiotic activity in different manners. The aminoglycosides might recognize different RNA conformations or the interaction might involve different RNA tertiary structures that are not equally sampled in our ribosome-free model. These results imply that models of ribosomal RNA must be carefully designed if the data are expected to accurately reflect biological activity.

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Introduction

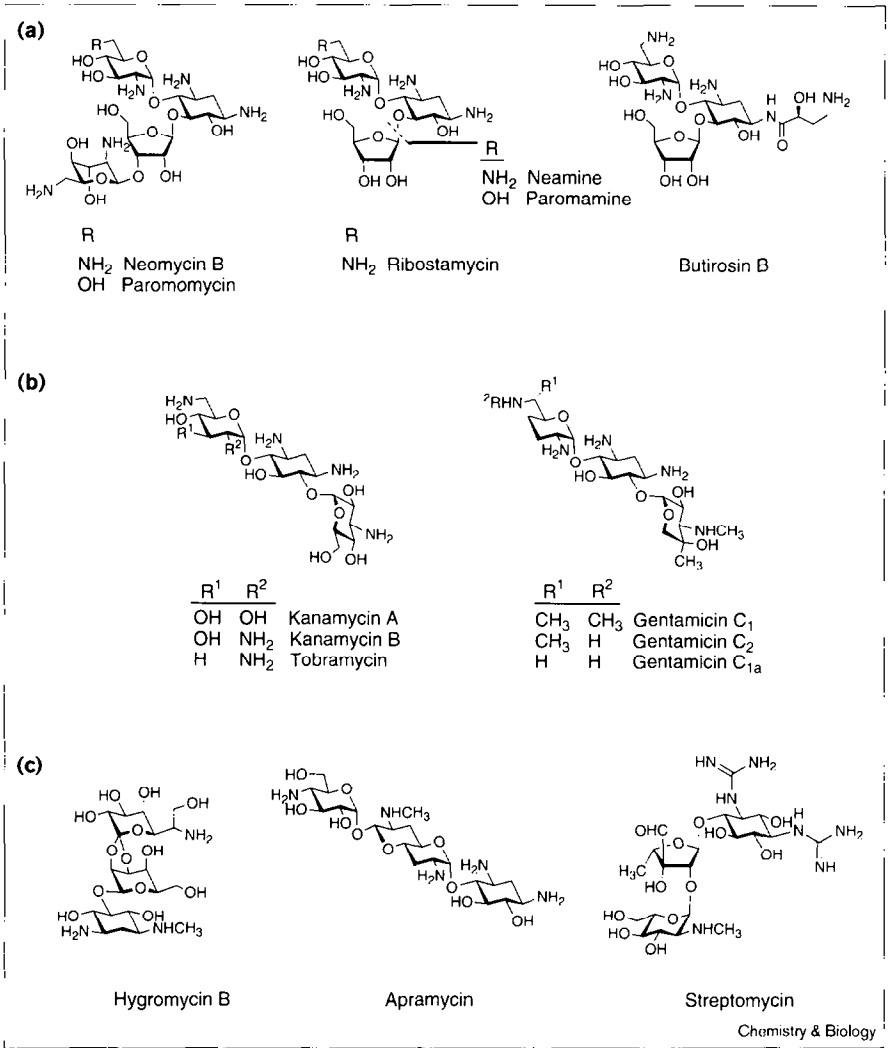
There is tremendous interest in developing small molecules that selectively target RNA because of their potential as therapeutic agents. Aminoglycosides [1] provide the best current lead structures because they have evolved to recognize specific sites within ribosomal RNA [2]. As a class, aminoglycosides have been known for five decades and the antibacterial properties of thousands of derivatives have been measured. At the molecular level, however, little is understood about the principles governing aminoglycoside–RNA recognition. Only recently, beginning with the pioneering work of Noller's group [3,4], have the properties of aminoglycosides been investigated at the RNA-binding level.

By far the largest number of aminoglycosides fall in the class that binds to the A-site of the ribosomal decoding region. The function of the A-site during protein synthesis is to bind the charged aminoacyl tRNA corresponding to the next mRNA codon in a transcript. In a mechanism that has yet to be clarified, the decoding region helps to ensure selection of the correct cognate tRNA, which is chosen with higher specificity than would be expected solely from the three base pair codon–anticodon interaction. Aminoglycosides that bind to the decoding region (Figure 1) interfere with the ribosomal 'proofreading' mechanism, and lead to miscoding and/or premature termination, as well as inhibiting nascent polypeptide translocation along the ribosome. These activities are believed to be the cause of the bactericidal action of these antibiotics [1].

The A-site-binding aminoglycosides include the 4,5-linked 2-deoxystreptamine derivatives neomycin B, paromomycin and ribostamycin, as well as the 4,6-linked kanamycins and gentamicins. The structurally dissimilar antibiotics hygromycin B and apramycin also belong to the A-site-binding group. One characteristic of all these A-site binders, in chemical footprinting experiments, is the strong protection of the G1494 N7 of the 16S ribosomal RNA. Otherwise, there are notable differences with respect to other footprints, as well as the observed patterns of antibiotic resistance. For example, methylation of the G1405 N7, which is found in the gentamicin producer strain *Micromonospora purpurea*, confers resistance to the 4,6-linked 2-deoxystreptamine derivatives gentamicin and kanamycin but not to neomycin B (a 4,5-linked aminoglycoside) [2].

The presence of ribosomal proteins is not necessary for binding of aminoglycosides to the decoding region, a phenomenon first demonstrated by Purohit and Stern [5]. They showed that a 50-nucleotide stem loop containing nucleotides corresponding to both the A-site and P-site of the decoding region binds to neomycin B or paromomycin

Figure 1



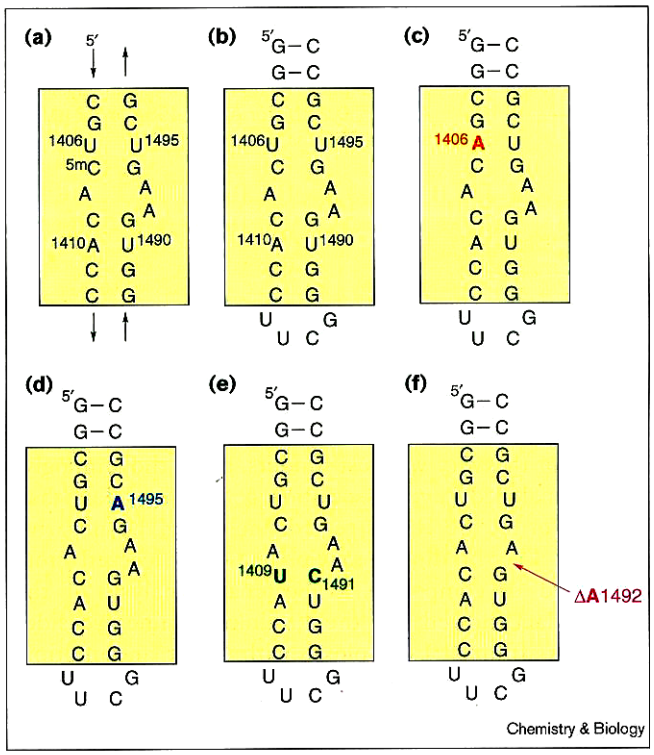
Structures of the aminoglycoside antibiotics used in this study. **(a)** The 4,5-linked 2-deoxystreptamine derivatives neomycin B, paromomycin, ribostamycin, neamine, paromamine, and butirosin B. Note that both neamine and paromamine are smaller two ringed structures, whereas ribostamycin is a larger structure. **(b)** The 4,6-linked 2-deoxystreptamine derivatives kanamycin A and B, tobramycin, and the gentamicins. **(c)** The structurally dissimilar aminoglycosides hygromycin B, apramycin, and streptomycin.

with high affinity, giving rise to a protection pattern similar to that observed for 16S ribosomal RNA in the context of the entire ribosome [5]. Later it was shown that an even shorter RNA hairpin (AS-wt), which corresponds to only bases of the A-site, contains an intact aminoglycoside-binding site [6,7]. Extensive mutational analysis has defined the nucleotides required for specific recognition of these antibiotics (Figure 2), which include a looped out nucleotide 1492, adjacent to a G–C base pair [6]. In the position corresponding to base 1495 in *Escherichia coli* 16S rRNA numbering either a uridine or a guanosine is required In order to close up the other side of the bulge a base pair is needed at position 1409–1491.

The structure of paromomycin complexed with the A-site model AS-wt RNA has been determined recently using nuclear magnetic resonance (NMR) spectroscopy [8]. Paromomycin sits in a pocket created by a bulged residue (A1492) and a non-canonical A1408–A1493 base pair (see Figure 2). The 2,6-diaminoglucose ring stacks against the underside of the C1409–G1491 base pair, which forms the floor of the bulge created by the looped-out adenosine residue and the non-canonical A1408–1493 base pair. The 2-deoxystreptamine ring spans two base pairs in the major groove and its two amino groups make specific hydrogen-bond contacts with the N7 of G1494 and the O6 of U1495. In addition, the N3 of the cyclitol ring and the hydroxyl groups of the glucosamine residue are involved in ionic- and/or hydrogen-bond interactions with the phosphates of A1493 and A1492. The other two rings of the bound aminoglycoside, the ribose and idose rings, are dynamic in structure and do not appear to be involved in any particular hydrogen bonds.

Aminoglycosides have a general affinity for RNA because of their multiple positive charges. More important from a medicinal perspective, however, is their binding specificity — that is, their ability to discriminate between different

Figure 2



RNA sequences related to the ribosomal decoding region A-site, which is shown in **(a)**. The five sequences include **(b)** the wild-type A-site RNA (AS-wt), **(c)** a positive-control mutant that improves neomycin binding (AS-U1406A), and three negative-control mutants: **(d)** AS-U1495A, in which a noncanonical U–U base pair is replaced with U–A; **(e)** AS-res, in which a C–G base pair is replaced with a U–C base pair; and **(f)** AS-ΔA1492, in which the bulged out A1492 is deleted.

RNA structures. The challenge of sequence- and structure-specific recognition of a particular target site against the backdrop of a largely homogeneous biopolymer is fundamentally important to the field of molecular recognition. Even proteins often show only moderate specificity (≤ 100-fold versus nonspecific sequences) in the recognition of RNA [9], underscoring the difficulties that are likely to be encountered in the design of specific small molecules to target RNA.

In addition to the interaction with their natural targets on the prokaryotic ribosome, aminoglycosides have been shown to bind a number of other different and diverse RNA sequences. These include two mRNA sequences from HIV, the Rev-responsive element (RRE) [10,11] and *trans*-activating response element (TAR) [12]. Aminoglycosides can also inhibit catalytically active RNAs such as the self-splicing group I introns [13,14] and the hammerhead ribozyme [15,16]. Other RNA sequences that bind to aminoglycosides have been derived using *in vitro* selection [17–21]. In many of these cases, however, the specificity of the interactions remains unknown, which is primarily due to the scarcity of methods available to address this issue. Recently, we developed a new method for directly observing aminoglycoside–RNA interactions based on surface plasmon resonance (SPR) [22]. Using SPR, we have investigated the recognition of the A-site of the ribosomal decoding region by aminoglycoside antibiotics.

Results and discussion

Specificity of paromomycin for A-site variants

To understand the specificity of ribosomal RNA recognition by the various aminoglycosides, we decided to investigate their binding to the AS-wt sequence and compare these results with those from a series of control mutants that have likewise been characterized using chemical footprinting (Figure 2). These negative controls include the AS-U1495A, AS-ΔA1492 and AS-res RNAs. The mutations found in these RNAs have been shown to abrogate specific binding to paromomycin [6]. In AS-U1495A a critical hydrogen-bond acceptor that interacts with N1 of the 2-deoxystreptamine ring has been removed, and the mutation ΔA1492 destroys the bulged pocket in which paromomycin ring II (the glucose ring) is positioned. Finally, in the AS-res mutant the G1491–C1409 base pair, which creates the floor of the binding pocket, is disrupted by replacing it with a C1491–U1409 mismatch base pair. Breaking up the critical G1491–C1409 base pair in the *E. coli* 16S ribosomal RNA causes resistance to a broad range of aminoglycoside antibiotics [2]. In contrast to these negative controls, the mutant hairpin AS-U1406A contains all the necessary elements for specific aminoglycoside recognition and binds paromomycin with wild-type specificity.

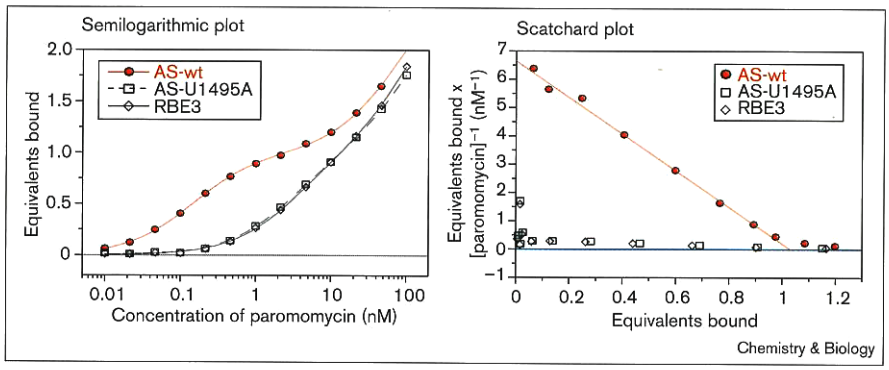
We utilized our recently developed SPR-based assay for quantitative measurement of binding of aminoglycosides to these sequences. Biotinylated derivatives of all sequences were prepared using *in vitro* transcription in the presence of guanosine 5′-monophosphorothioate followed by alkylation with a biotin iodoacetamide derivative [22]. The RNA conjugates were then immobilized onto streptavidin-coated SPR sensorchips for analysis.

Figure 3 shows the results for binding of paromomycin to AS-wt. A clear 1:1 binding isotherm is visible at low concentrations, which is overlaid with nonspecific binding to additional equivalents at higher concentrations. The paramomycin–AS-wt binding curve is in contrast with the curve obtained for the negative-control mutant AS-U1495A (which binds paromomycin 20-fold more weakly than AS-wt) because it does not show formation of any discernible 1:1 complex.

Binding of aminoglycoside antibiotics to A-site variants

In the same manner as described above for paromomycin, a panel of aminoglycosides was screened and the results are compiled in Table 1. Each dissociation constant was

Figure 3



Paramomycin-specific binding to AS-wt compared with negative-control RNAs AS-U1495A and RBE3 (a completely unrelated RNA hairpin). The K_d value of paramomycin for AS-wt is 0.2 μM, for AS-U1495A is 2.7 μM and for RBE3 is 3.2 μM.

determined from 1–3 independent trials. In general, we have found that the SPR assay results have good reproducibility and, based on the observed variation in multiple determinations, we estimate that all K_d values are accurate within a factor of three. Importantly, it should be noted that relative affinities of a given aminoglycoside for different RNA sequences are very reliable, as they are determined simultaneously in a single SPR experiment using the same solution of ligand. All data were recorded under near-physiological salt conditions (in HBS buffer: 150 mM NaCl, 10 mM HEPES, pH 7.4 and 3 mM EDTA) unless noted otherwise. Although magnesium might be expected to influence aminoglycoside binding to RNA, it is known that increasing the Mg^{2+} concentration from 0 to 10 mM does not affect binding to these sequences [6]. Data points were taken at aminoglycoside concentrations ranging from 0.01 to 100 μM. Below this concentration range, surface-transport limitations prevent the system from attaining equilibrium.

Table 1

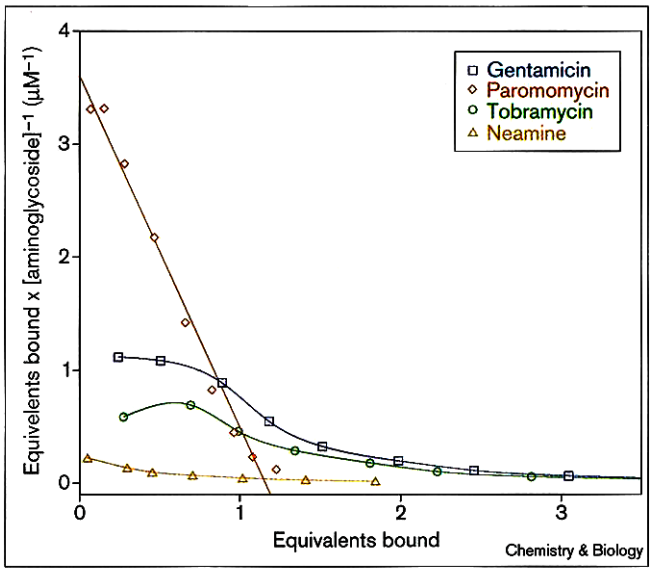
Binding of aminoglycosides to variants of the ribosomal A-site decoding region.

Compound	AS-wt	U1406A	U1495A	AS-res	ΔA1492
Neomycin B	0.019	< 0.01	0.38	0.48	0.32
Paromomycin	0.20	0.027	2.7	5.7	5.7
Kanamycin B	1.4	4.4	4.0	3.5	2.7
Tobramycin	1.5	2.1	4.1	7.9	4.5
Gentamicin	1.7	9.9	12	18	16
Apramycin	6.3	9.3	13	NA	NA
Ribostamycin	25	11	90	52	38
Kanamycin A	18	28	33	37	32
Neamine	7.8	5.5	31	NA	NA
Butirosin	27	1.8	99	NA	NA
Paromamine	> 100	> 100	> 100	> 100	> 100
Hygromycin B	> 100	> 100	> 100	> 100	> 100
Streptomycin	94	66	74	NA	NA

All values are dissociation constants in units of μM. NA, not available.

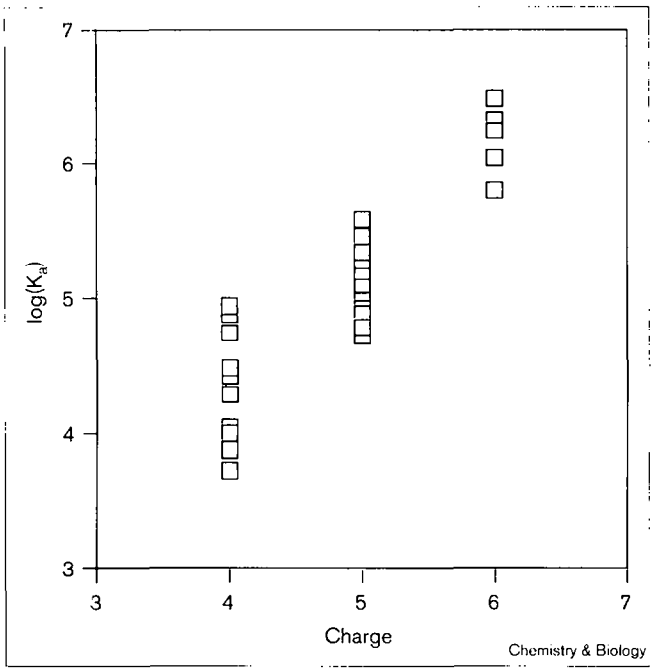
Except for the very weakest binders, all of the aminoglycosides analyzed have dissociation constants of less than 100 μM for all the RNAs tested, including the negative controls. The binding behavior, however, varied from compound to compound. A Scatchard analysis of the binding data showed that the 4,5-linked aminoglycosides form high-affinity 1:1 complexes with AS-wt, and multiple equivalents bind only at higher relative concentrations of aminoglycoside. The 4,6-linked aminoglycosides did not appear to form clear 1:1 initial complexes, however. Rather, there appear to be multiple equivalents of aminoglycoside binding with similar affinity. Figure 4 illustrates the binding of paromomycin, a 4,5-linked aminoglycoside, tobramycin and gentamicin from the 4,6-linked class, and

Figure 4



Scatchard analysis of binding to AS-wt by the 4,5-linked aminoglycoside paromomycin, the 4,6-linked aminoglycosides gentamicin and tobramycin and the core structure neamine.

Figure 5



Relationship between the number of amines (charge) in each aminoglycoside and their respective nonspecific binding affinity (K_a). The number assigned for charge refers to the fully protonated state. Note that with each added charge, nonspecific affinity increases approximately tenfold.

neamine (the structure of which forms the basis of both the 4,5- and 4,6-linked classes). Neamine appears to have a second binding event at a slightly higher concentration than the initial 1:1 complex, as its Scatchard plot is not absolutely linear.

The compounds in Table 1 are grouped according to the number of positive charges at full protonation. Due to the low pK_a of some amino groups within the aminoglycosides (e.g., N3 of neomycin B has a $pK_a < 6$) [23], the actual protonation state at pH 7.4 will be lower than expected. Nonetheless, a clear trend can be seen when examining the charge dependence of aminoglycoside binding to the negative-control mutant RNAs, AS-U1495A, AS-res and AS-ΔA1492 (Figure 5).

Depending on the number of amines, nonspecific binding is in the 1, 10 or 100 μM range for compounds with six, five or four amino groups, respectively. The general trend of an increase in binding affinity by a factor of ten for every added charge also applies when comparing compounds that bind specifically to the A-site (e.g., paromomycin and neomycin B). These results are consistent with recent observations by Wang and Tor [24] in which synthetic aminoglycoside derivatives with additional charges were more active in binding and inhibiting the hammerhead ribozyme.

The affinities of the aminoglycosides for the three different negative controls showed little variation. Comparison of the binding data for AS-wt with these values reveals the recognition specificity of the aminoglycosides for the A-site sequence. The 4,5-linked compounds with a 2-deoxystreptamine core (neomycin B, paromomycin, ribostamycin) are all specific for AS-wt over the negative-control RNAs. Within this group, four-ringed compounds show the tightest affinities and highest specificities, and binding to the positive-control AS-U1406A is also enhanced. In contrast, the specificity of the AS-U1406A mutant for ribostamycin is lower and binding is only weakly enhanced. Butirosin, which is a trisaccharide carrying a 4-amino-2-hydroxy-butyric acid substituent on N1, likewise shows only moderate specificity for AS-wt, but, unlike ribostamycin, binding to AS-U1406A is significantly enhanced.

The 4,6-linked 2-deoxystreptamine derivatives (kanamycin A, kanamycin B, tobramycin and gentamicin) have only very moderate specificity for AS-wt. Furthermore, unlike the 4,5-linked compounds, their binding affinity to AS-U1406A relative to AS-wt is decreased. Both groups of aminoglycosides considered here, the 4,5- and 4,6-linked 2-deoxystreptamine derivatives, are derived from the same core structure (based on neamine), and share a common disaccharide motif. Neamine has increased nonspecific binding affinity compared to other compounds with four amines. Like ribostamycin, neamine still retains moderate specificity for AS-wt over the negative-control AS-U1495A. Removing the 6′-amino group of neamine (paromamine) leads to a substantial decrease in binding to AS-wt and a loss of specificity for the A-site RNA sequence.

Apramycin shows only marginal, if any, specificity for AS-wt and hygromycin B shows none. Both compounds are known to be A-site binders based on chemical footprinting data, but they are structurally dissimilar to the neamine-containing group of antibiotics. Structurally, it is clear that neither apramycin nor hygromycin B can interact with AS-wt in the same way as paromomycin. It is therefore likely that AS-wt is not an appropriate sequence for examining binding of these two compounds to ribosomal RNA. Finally, streptomycin, which binds a different region of the ribosome not involving the A-site, does not show any specificity for AS-wt (as expected).

Table 2 shows a comparison of the binding specificities in the 4,5- and the 4,6-linked series. The average K_d value for nonspecific binding was calculated from the binding constants to the negative controls AS-U1495A, AS-res and AS-ΔA1492.

For the neomycin series (i.e., the 4,5-linked 2-deoxystreptamine derivatives) the same order of specificities as seen for

Table 2

Specificity of recognition of AS-wt and AS-U1406A by various aminoglycosides.

Compound	Average K_d(nonspecific) (μM)	Specificity for AS-wt	Specificity for U1406A
4,5-linked			
Neomycin B	0.39	20	>40
Paromomycin	4.7	20	200
Butirosin	99	4	60
Neamine	31	4	6
Ribostamycin	60	2	5
4,6-linked			
Gentamicin	16	9	2
Tobramycin	5.5	4	3
Kanamycin B	3.4	2	~1
Apramycin	13	2	1
Kanamycin A	34	2	1
Control			
Streptomycin	74	~1	1

The K_d(nonspecific) was compared to both K_d(AS-wt) and K_d(AS-U1406A) to derive the specificity values.

AS-wt is observed for the positive-control AS-U1406A, but the magnitude of the specificity is increased. An entirely different situation is encountered with the 4,6-linked series (kanamycins, tobramycin, gentamicin) in which the observed specificities, with the exception of gentamicin, are very moderate and practically no specificity is seen for the binding of these compounds to the AS-U1406A.

Although the amount of specificity varies for each aminoglycoside, in every case binding is worse for the AS-res mutant, which confers aminoglycoside resistance in *E. coli* [2], than for AS-wt (Table 1). This observation underscores the need to target such mutations with new compounds to counter the emergence of aminoglycoside-resistant bacterial strains. We would ultimately like to develop compounds that do not have specificity for AS-wt over sequences such as AS-res.

Influence of ionic strength and pH

Both ionic strength and pH influence aminoglycoside binding, as shown in Table 3. Raising the pH from 7.4 to 7.8 has a modest effect of decreasing binding but, as judged from the ratio of K_d(AS-U1495A) to K_d(AS-wt), the specificity remains unchanged. Increasing the amount of competing ions by adding ammonium chloride likewise decreases both the specific (AS-wt) and the nonspecific (AS-U1495A) binding to paromomycin or neomycin B. The effect is greater for nonspecific binding, however. Thus, the specificity of both compounds for AS-wt increases at higher salt concentrations, an observation that agrees with the general notion that charge–charge interactions are the main, if not only, driving force for nonspecific RNA binding and that specific recognition involves additional, nonionic contributions.

Table 3

Influence of pH and ionic strength on the specificity of RNA recognition.

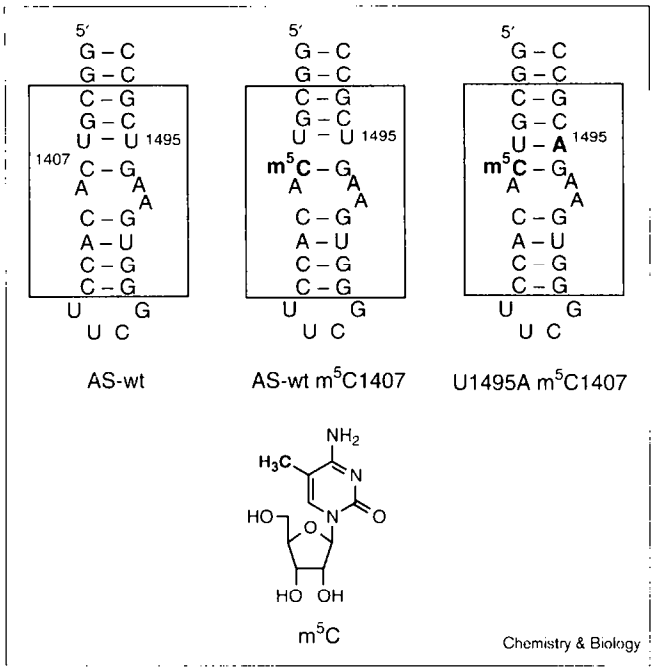
Compound	AS-wt	AS-U1495A	Specificity for AS-U1495A
Paromomycin			
HBS buffer alone	0.20	2.7	14
+ 50 mM NH_4Cl	0.29	6.5	23
+ 150 mM NH_4Cl	1.1	32	28
pH 7.8	0.53	7.7	15
Neomycin B			
HBS buffer alone	0.019	0.38	20
+ 50 mM NH_4Cl	0.025	1.1	43
+ 150 mM NH_4Cl	0.15	6.7	43
pH 7.8	0.044	0.91	21

Values of dissociation constants are measured in μM.

Effect of RNA methylation

Although the chemical footprints on the intact ribosome for the 4,6-linked compounds overlap the footprints for neomycin B and paromomycin, the binding orientation of the molecule could well be different, as suggested by the poor binding to the positive-control AS-U1406A mutant. An intriguing possibility is that the naturally occurring methylation on C1407 might be required for recognition by these compounds. We addressed this possibility using A-site RNAs containing this modification. *E. coli* 16S ribosomal RNA contains several modified nucleosides, among them three located within the decoding region, N^4,2′-*O*-dimethylcytidine 1402, 5-methylcytidine (m^5C) 1407, and 3-methyluridine 1498 [25–27]. Of these, m^5C1407 is located within the aminoglycoside-binding domain of the A-site. Inspection of the NMR solution structure of paromomycin bound to AS-wt [8] reveals that the methyl substituent of m^5C1407 would be juxtaposed to the hydrophobic α face of the 2,6-diamino-2,6-dideoxy-L-idose ring of the aminoglycoside. Depending on the precise atomic positions, this could be either a stabilizing hydrophobic interaction, due to exclusion of water from the interface, or a destabilizing steric clash. In addition, molecular modeling based on the NMR structure suggests that ring 3 of 4,6-linked aminoglycosides, such as gentamicin or tobramycin, could also contact the methyl substituent of m^5C1407. It is already known that this base modification does not adversely affect aminoglycoside binding, as Noller and colleagues' footprinting data [3,4] was generated on whole ribosomes containing m^5C1407, but the possibility that binding could be enhanced remained untested. Because our initial enzymatically synthesized RNA oligonucleotides (see above) contained cytidine rather than the natural m^5C at position 1407, we prepared several chemically synthesized RNA oligonucleotides in order to study the effect of a 5-methyl substituent at position 1407 on aminoglycoside binding.

Figure 6



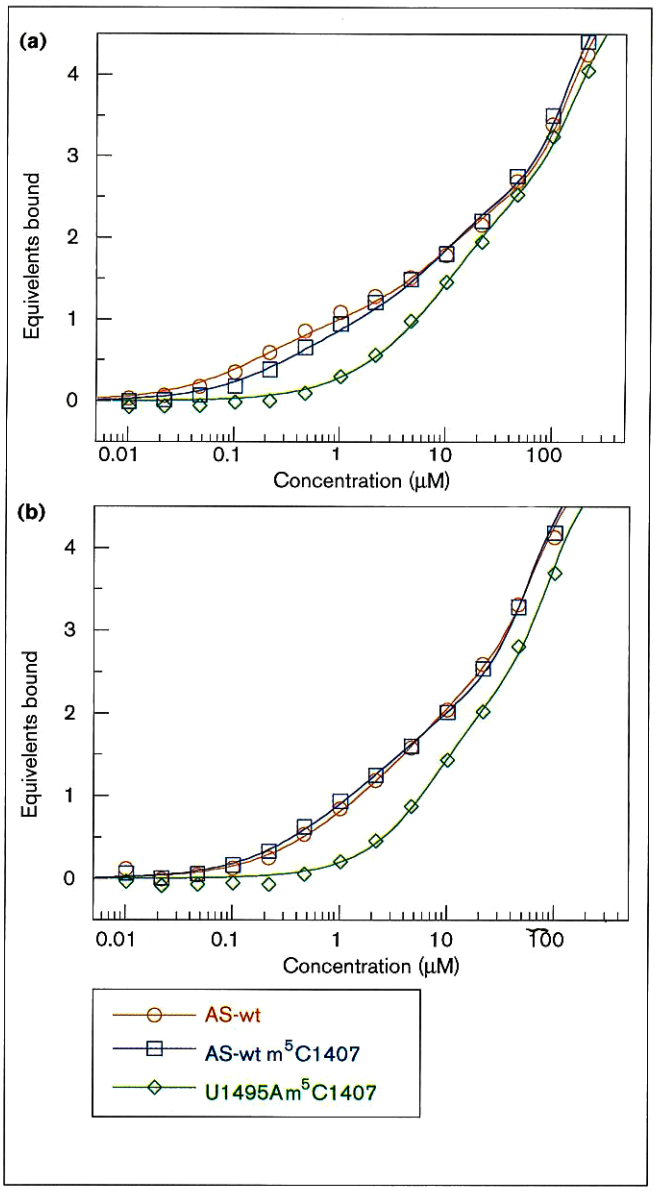
RNA sequences used in probing the influence of methylation of C1407. This base modification is found in *E. coli* ribosomal RNA, and potentially could affect aminoglycoside binding to the decoding region A-site.

The sequences of 5′-biotinylated oligoribonucleotides AS-wt, AS-wt m^{5}C1407, containing the m^{5}C1407 substitution, and U1495A m^{5}C1407, containing the deleterious U1495A mutation along with the m^{5}C1407 substitution, are shown in Figure 6. The oligoribonucleotides were chemically synthesized using commercially available 5-methyl-*N*^{4}-benzoyl-5′-*O*-(dimethoxytrityl)-2′-*O*-(*t*-butyldimethylsilyl)cytidine-3′-*N*,*N*-diisopropyl(cyanoethyl) phosphoramidite, gel purified, and immobilized on a streptavidin-coated SPR sensor chip surface. Aminoglycoside binding experiments in HBS buffer at 25°C or 37°C were performed as described above.

Figure 7 shows representative binding data and derived isotherms for paromomycin and gentamicin binding to the various RNA sequences. These data show that the m^{5}C1407 substitution has little effect on either paromomycin or gentamicin binding. The equilibrium dissociation constants derived from a series of binding experiments with paromomycin, tobramycin, and gentamicin are shown in Table 4. For paromomycin, the K_d for AS-wt m^{5}C1407 is twofold less than AS-wt, a difference that approaches the limit of accurate measurement. For both paromomycin and gentamicin, the K_d values for AS-wt and AS-wt m^{5}C1407 are identical.

These results suggest that there is no interaction between the 5-methyl substituent of m^{5}C1407 and the

Figure 7



Semilogarithmic plots of paromomycin and gentamicin binding to AS-wt, AS-wt m^{5}C1407, and U1495A m^{5}C1407.

aminoglycoside antibiotics studied. Clearly, an unfavorable steric clash can be ruled out, as this would be expected to reduce binding affinity significantly for both the AS-wt m^{5}C1407 and the U1495A m^{5}C1407 sequences. It is also clear that an optimized hydrophobic contact is not occurring. In the case of the 4,5-linked aminoglycosides, the NMR structure indicates considerable conformational mobility for the 2,6-diamino-2,6-dideoxyidose ring. The conformation shown in the NMR structure, which juxtaposes the idose ring with the methyl group, might not be the active conformation of the RNA *in vivo*. If this conformation occurred only a small fraction of the

Table 4

Equilibrium dissociation constants (μM) for aminoglycoside binding to AS-wt, AS-wt m^{5}C1407, and U1495A m^{5}C1407.

Aminoglycoside	AS-wt	AS-wt m^{5}C1407	U1495A m^{5}C1407
Paromomycin	0.18	0.38	3.0
Gentamicin	0.61	0.50	5.9
Tobramycin	1.0	1.2	5.0

time, the effects of any potential interaction would be ameliorated. In the case of the 4,6-linked aminoglycosides, it is possible that ring 3 does not actually contact m^{5}C1407. Because there is no structural data available in this case, the possibility cannot be ruled out.

To evaluate the validity of the binding data obtained through SPR, it is instructive to compare the results obtained here with binding constants obtained using other experimental techniques. Puglisi and coworkers [6] have determined the dissociation constant of the paromomycin–AS-wt complex at 0.2 μM using quantitative chemical footprinting. Their value is in excellent agreement with the dissociation constant obtained using SPR (0.2 μM). Working with a slightly extended RNA hairpin containing the same wild-type sequence, Purohit and Stern [5] obtained RNase footprints that suggest a binding constant of ~0.1 μM for neomycin B, compared with 0.02 μM determined using our assay. Using a competition fluorescence assay, Rando and coworkers [28] determined dissociation constants with a longer RNA hairpin containing the A-site. These workers obtained K_d values for paromomycin (1.9 μM), neomycin B (0.13 μM), gentamicin (1.8 μM), kanamycin B (2.1 μM) and tobramycin (1.7 μM) [28]. Thus, the same tenfold difference in binding affinity between paromomycin and neomycin B is observed by these authors, and the differences in the determined K_d values versus the SPR assay is within an order of magnitude for all compounds. Similar affinities have also been obtained when studying ribosomal binding, rather than the binding of isolated RNA. Both kanamycin [29] and tobramycin [30] bind to intact ribosomes with an affinity of 1–10 μM as determined by equilibrium dialysis [31]. There is, therefore, good qualitative and quantitative agreement of the binding data obtained using SPR with previously determined RNA-binding data. Compared to gel-based assays, the SPR assay is quicker, generally more reliable for small-molecule–RNA interactions [22], and allows determination of binding stoichiometry.

Recently we reported on the A-site binding and antibacterial activity of a number of synthetic derivatives of neomycin and some of the natural 4,5-linked aminoglycosides [32]. A synthetic derivative with lower binding affinity and specificity than neomycin had equivalent antibacterial activity, suggesting that factors that cannot be addressed using this RNA model system are important for activity. This is probably also the case for the 4,6-linked compounds reported here, as they are clinically effective antibiotics despite the much lower affinity and specificity observed in the AS-wt model system.

Also very recently, Puglisi and coworkers [33] reported NMR and chemical-modification studies of the 4,5-linked aminoglycosides neomycin, paromomycin, and ribostamycin, as well as neamine. Their studies suggest that this class binds A-site RNA specifically, and with similar structures. These results are consistent with those we have obtained using SPR. A correlation between our very different data on the 4,6-linked aminoglycosides and structural characterization of a complex of one of these compounds with A-site RNA remains to be determined.

We believe that the results presented here reflect the fact that protein-free ribosomal RNA model systems are taken out of their ribosomal context. As such they lack geometric constraints imposed by the ribosomal surroundings, which might, or might not affect ligand binding. Clearly, AS-wt is a good model system for studying the interaction between the ribosomal A-site with the 4,5-linked aminoglycosides. In this regard it is likely that the positive control mutant AS-U1406A approximates the conformation of the bound ribosomal complex even better than AS-wt and is therefore the preferred model for developing structure–activity relationships for these aminoglycosides.

On the other hand, AS-wt appears to be a less accurate model for the binding behavior of the other major class of clinically important aminoglycosides, namely the 4,6-linked trisaccharides kanamycin A, kanamycin B, tobramycin and gentamicin. This could be due to the fact that the 4,6-linked class recognizes a different conformation of the same RNA sequence that is not well represented in the AS-wt model, or because tertiary interactions with other parts of the ribosome are important for binding by this class of antibiotics. Both classes of aminoglycosides bound with lower relative affinity to AS-res, a sequence derived from aminoglycoside-resistant *E. coli*. Such mutant sequences are an important target for design of new antibiotics to combat resistant bacteria.

Care must be taken in the selection of the proper *in vitro* RNA model system, as seen from the results for the A-site models. The available structural evidence from NMR suggests that short RNA hairpins are quite disordered in the absence of a bound ligand. This dynamic behavior will impose energetic penalties that could obscure binding events of moderate specificity. In the case of ribonucleoprotein complexes such as the ribosome, protein-free models that appear to bind ligands similarly to the parent system (by chemical footprinting) might not accurately reflect the *in vivo* binding event. Although it is believed

that aminoglycosides solely contact RNA in the ribosome, and not protein, it is likely that local RNA–protein tertiary interactions affect ligand binding and vice versa. With this in mind, we believe that binding of aminoglycosides to their ribosomal target sites will probably not follow a simple 'competitive inhibition' paradigm. Rather, a bound aminoglycoside molecule allosterically interferes with protein synthesis by lowering the selectivity of cognate versus noncognate aminoacyl-tRNA selection, as has been suggested for streptomycin and neomycin [34]. Conceptually, this is more akin to a receptor signaling event, where a bound ligand irrespective of its affinity may either behave as an agonist or an antagonist. It is not at all clear whether increased affinity of a ligand for the ribosomal A-site would translate into better potency as an antibacterial drug, which is especially noteworthy when considering that the ribosome is present at micromolar concentrations in the cell.

Significance

Although aminoglycoside antibiotics have been in use for over 50 years, only recently have we begun to understand their mechanism of action on a molecular level. We have used surface plasmon resonance (SPR) to study the interactions of a number of structurally diverse aminoglycoside antibiotics with RNAs that model the *E. coli* ribosomal decoding region A-site RNA. SPR allows rapid quantitative determination of the binding constant and stoichiometry, and is more reliable than gel assays for studying small-molecule–small-RNA interactions. Specificity for the wild-type sequence over closely related variants was observed for the 4,5-linked 2-deoxystreptamine derivatives that comprise the neomycin class of aminoglycosides. The 4,6-linked derivatives of the kanamycin/gentamicin class, and other structurally dissimilar aminoglycosides, that have been footprinted to the same ribosomal-binding site, had different, and weaker, specificity profiles toward these RNA sequences. The results suggest that the two aminoglycoside classes bind the A-site in different manners, possibly involving tertiary interactions that cannot be accounted for in this ribosome-free model system. They may effect their antibiotic activity through subtly different changes to the conformation and dynamics of the ribosomal A-site. The results imply that care must be taken in designing *in vitro* model systems for studying RNA–small-molecule interactions if results are expected to accurately reflect biological activity.

Materials and methods

Neomycin B sulfate (Fluka) was converted to the free base by passing it through Amberlite IRA 400 (OH- form) and purified by ion exchange chromatography on Dowex 1-X2 100; the purity of neomycin B was verified by NMR in D_2O. Neamine was obtained by acid-catalyzed cleavage of neomycin B and purified by ion exchange chromatography on Amberlite CG-50. Paromamine was obtained by acid-catalyzed cleavage of paromomycin and purified in the same manner. Paromomycin sulfate, ribostamycin sulfate, kanamycin A sulfate, kanamycin B sulfate, butirosin sulfate and streptomycin sulfate were obtained from Sigma and used as received. Tobramycin, gentamicin sulfate (gentamicin C complex containing a mixture of the structures shown in Figure 1), apramycin sulfate, and hygromycin B hydrochloride were obtained from Fluka and used as received.

RNA Synthesis

Enzymatically synthesized 5-biotinylated RNAs were prepared as previously described [22]. Synthetic RNAs containing the m^5C modification were prepared as follows. N^6-phenoxyacetyl-5′-*O*-(dimethoxytrityl)-2′-*O*-(*t*-butyldimethylsilyl)-adenosine-3′-*N,N*-diisopropyl(cyanoethyl) phosphoramidite, N^2-isopropylphenoxyacetyl-5′-*O*-(dimethoxytrityl)-2′-*O*-(*t*-butyldimethylsilyl)guanosine-3′-*N,N*-diisopropyl-(cyanoethyl) phosphoramidite, N^4-acetyl-5′-*O*-(dimethoxytrityl)-2′-*O*-(t-butyldimethyl-silyl)cytidine-3′-*N,N*-diisopropyl(cyanoethyl) phosphoramidite, 5′-*O*-(dimethoxytrityl)-2′-*O*-(t-butyldimethylsilyl)uridine-3′-*N,N*-diisopropyl(cyanoethyl) phosphramidite, and cytidine-derivatized polystyrene support were purchased from Pharmacia. 5-methyl-N^4-benzoyl-5′-*O*-(dimethoxytrityl)-2′-*O*-(*t*-butyldimethylsilyl)cytidine-3′-*N,N*-diisopropyl(cyanoethyl) phosphoramidite was purchased from Chemgenes and 5′-biotin phosphoramidite was purchased from Glen Research. All phosphoramidites were used as 0.1 M solutions in anhydrous acetonitrile. Oligoribonucleotides were synthesized on a Pharmacia Gene Assembler Special using 0.25 M 5-ethylthio-1*H*-tetrazole as the activator and 5 min coupling times for all phosphoramidites [34]. After synthesis, the solid supports were vacuum dried and treated with ammonia saturated ethanol for 18 h. The supernatants were removed, divided into four equal aliquots, and lyophilized. These aliquots were then each treated with 250 µl of deprotection solution (0.75 ml *N*-methylpyrollidinone). 375 ml triethylamine, and 0.5 ml triethylamine•3HF) and incubated for 1.5 h at 65°C. The deprotected oligos were precipitated by addition of 25 µl 3M sodium acetate, followed by 1 ml *n*-butanol. After centrifuging for 15 min at 14,000 rpm, the supernatant was removed, and the pellet was washed twice with 70% ethanol and vacuum dried. The crude oligonucleotides were purified by electrophoresis on denaturing 20% polyacrylamide gels, recovered by the 'crush and soak' method, and desalted on NAP-10 size exclusion columns (Pharmacia) in 1 mM Tris, pH 7.5, 0.1 mM EDTA. The resulting oligonucleotide solutions were stored at −20°C.

Binding experiments

Binding experiments were performed on a BIAcore 2000 instrument essentially as previously described [22]. Sensor chips SA (BIAcore AB) were preconditioned by washing with three 1 min pulses of 1 M NaCl, 50 mM NaOH at 10 µl/min. 0.1 µM RNA solutions were renatured by heating to 85°C and slowly cooling to ambient temperature. The solutions were adjusted to 1M NaCl, 0.5X HBS before immobilizing on the sensorchip at a flow rate of 10 µl/min for 5 min. Aminoglycoside samples were prepared by serial dilutions from stock solutions in RNase free microfuge tubes (Ambion) and were centrifuged at 14000 rpm for degassing. Unless otherwise noted, all binding studies were carried out using HBS buffer (Pharmacia Biosensor AB) which was used as obtained. All procedures for binding studies were automated as methods using repetitive cycles of sample injection and regeneration. Typically, buffer was injected in the first two cycles to establish a stable baseline value. Samples were injected at a flowrate of 5–10 µl/min using the KINJECT command. To minimize carry over, samples were injected in order of increasing concentration. Each data point was corrected for long term instrument drift by comparison of baseline values in an unfunctionalized reference flow cell with the original value prior to the experiment. Expected values for the equilibrium response of one equivalent of analyte were calculated from the relative molecular weight of the analyte and the immobilized RNA ligand in each flowcells and adjusted with a correction factor of 0.76 which arises from the different molar refractive indices of RNA and the analyte. Binding constants were calculated by fitting the data (equivalents of aminoglycoside bound vs. aminoglycoside concentration) to a model with n independent constants using the fitting program provided in the program Kaleidagraph (Macintosh). Data from two independent experiments were averaged to obtain the reported dissociation constants.

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