Aminoglycoside–RNA interactions
Frank Walter, Quentin Vicens and Eric Westhof*

The structural and physico-chemical parameters promoting the binding of aminoglycosides to RNAs are becoming clear. The strength of the interaction is dominated by electrostatics, with the positively charged aminoglycosides displacing metal ions. Although aminoglycosides inhibit most known ribozymes, aminoglycosides or polyamines are able to catalyze specific RNA cleavage in the absence of metal ions.

Addresses
Institut de Biologie Moléculaire et Cellulaire du CNRS, UPR 9002, 15 rue René Descartes, F-67084 Strasbourg, France
*e-mail: westhof@ibmc-u-strasbg.fr

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Abbreviations
A-site acceptor site
HDV hepatitis delta virus
$K_d$ dissociation constant
$K_i$ inhibition constant
TAR transactivating response element

Introduction
The potential of RNA as a new drug target has recently come to the fore [1–3], with the recognition that RNA molecules can adopt complex three-dimensional structures that, as with proteins, enable the design of specific ligands. Another reason for the present interest comes from the fact that many pathogenic agents, such as retroviruses, encode their genetic information in RNA strands. Aminoglycoside antibiotics (Figure 1) have long been used as very efficient drugs against Gram-positive and Gram-negative bacteria, and against mycobacterial infections [4]. These molecules, however, impair hearing and kidney functions at high doses and resistant strains are appearing at an increasing rate [5]. Thus, the need for new and more specific compounds has emerged. To enable the development of new antibiotics, it is necessary to understand at the molecular level the interactions between aminoglycosides and RNA, often considered as a model system. Interactions between RNAs and aminoglycosides have been reviewed in the past (see [1,6–9]). We center this review on papers published in the past two years.

Figure 1 legend
The antibiotics of the aminoglycoside family result from modifications of neamine, a two-ring system comprising 2-deoxystreptamine (ring II) glycosylated at the 4-position by a six-membered aminosugar (ring I) of the glycopyranoside series. Further modifications of ring II with various aminosugars at the 5-position lead to (a) the neomycin family, and at the six-position to (b) the gentamicin and (c) the kanamycin families. Amino groups of the aminoglycosides protonated at pH 7.0 are shown with $pK_a$ values, where known. The rings are flexible by torsional rotations about the glycosidic bonds; rotations of ring I about ring II are more hindered than those of ring II about ring I [7]. Aminoglycosides are polycationic in character. The protonated amino groups can serve, in addition to specific hydrogen bonds and van der Waals contacts, as donor groups making complementary electrostatic interactions with the electronegative density created by the fold of the polyanionic RNA backbone. In addition, the hydroxyl groups, because of their water-like behavior, can replace water molecules and form hydrogen bonds with anionic phosphate oxygens and the heterocyclic atoms of the bases. Ion and water displacement should give favourable entropic contributions to the binding strength.

Binding of aminoglycosides to natural RNAs
Ribosomal RNA
NMR experiments revealed the first atomic insights into complexes between aminoglycosides of the neomycin family (Figure 1a) and a 27 nucleotide stem–loop fragment incorporating the acceptor site (A-site) of 16S rRNA (dissociation constant $[K_d] = 0.2 \mu M$) [10,11 ••,12 ••]. It appears that both rings I and II of the aminoglycoside are important for its binding to an A-A mismatch adjacent to a bulging adenine. These two rings (though possessing different side groups) are oriented in the same way even in the complexes, with the additional rings (ring III for ribostamycin and rings III and IV for neomycin or paromomycin) contributing to an increase in the affinity of the binding of the antibiotic to the A-site. The gentamicin group of aminoglycosides (Figure 1b) has also been studied [13••]. These antibiotics, like the kanamycin family (Figure 1c), contain both rings I and II, but with two hydroxyl groups missing on ring I, and a different ring III linkage to that of the neomycin family. The gentamicin binds to the A-site with a $K_d = 2 \mu M$. Rings I and II occupy the same positions in the RNA pocket as the aminoglycosides of the neomycin family (Figure 2) formed by the three adenines (root mean square deviations for rings I and II are 1.28 Å and 0.41 Å, respectively). Because of the difference of the linkage, ring III of gentamicin interacts with the upper part of the RNA stem, whereas in the case of the paromomycin, rings III and IV line the deep groove in the lower part of the stem. The results observed by NMR are in each case strongly supported by biochemical tests [14 ••] and mutational studies [15 ••]. It has been suggested [16•] that the observed differences in specificity and binding strengths between the neomycin and kanamycin aminoglycosides [17•] could be attributable to the presence of several binding modes for the smaller kanamycin systems. In support of this analysis, NMR studies [12••] show that unsubstituted neamines (from the neomycin family) bind in alternative orientations. A surface plasmon resonance study indicated that the aminoglycosides lacking
A-site 16S rRNA loop.

transferred to another stem-loop structure based on the coside affinity for the other site. The recognition of the not bind by itself but indirectly increases the aminoglycoside recognition site is indicated.

µneomycin B and ~2

Gentamicin C1a

Paromomycin

Superimposition of the neamine moieties of paromomycin and gentamicin C1a in their NMR-determined complexes with the ribosome A-site [10,14**]. Ring III in each of the two antibiotics occupies a different region in space. In both complexes, rings I and II of the neamine make shape-sensitive contacts to a Watson–Crick and an A–A non-Watson–Crick pair, whereas rings III and IV form shape-insensitive electrostatic interactions with negatively charged regions around the opposite sugar-phosphate backbone. The ring structures are labelled with Roman numerals, and positions on ring II are indicated.

rings III and IV of the neomycin group and the kanamycin family have weaker specificity profiles towards the A-site RNA [17*] than neomycin. Paromomycin is a non-competitive inhibitor of tRNA, suggesting a simultaneous binding of the aminoglycoside and the tRNA. However, despite the binding of paromomycin in the deep groove of the A-site, a three-dimensional model of the tRNA–mRNA complex binding to the deep groove of the A-site has been proposed in agreement with all the available data [18].

Viral RNA

Two essential elements of the HIV genome, the rev-response element and transactivating response element (TAR) RNA motifs, bind aminoglycosides [21,22]. A detailed overview is given elsewhere [7]. It is important, however, to emphasize that neomycin behaves as a non-competitive inhibitor of Tat (the cognate protein) binding to TAR; neomycin displaces the Tat protein by increasing the $K_d$ of the peptide via an allosteric mechanism [23•].

Binding of aminoglycosides to in vitro selected RNA

RNA aptamers binding to aminoglycosides with high affinity (in the nanomolar range) have also been selected by in vitro methods. The structures of the aptamers complexed with small molecules have been extensively reviewed [8,24–26]. The NMR solution structures of two tobramycin-binding aptamers have been solved [27,28**], as well as that of the neomycin B binding aptamer [29**]. Interestingly, all aptamers have a similar architectural fold but little sequence homology (Figure 3). In each case, the neamine unit is deeply buried in a hairpin at the interface between a widened deep groove and a conserved six-residue loop from which a base loops out and covers rings I and III. The widening of the deep groove is caused by a bulge base (aptamer I against tobramycin) or by a zippered-up internal loop of three mismatches (aptamer II against tobramycin and neomycin aptamers). The 2′-amino and 6′-amino groups of ring II of neomycin and the 1 and 3 amino groups of ring I form potential hydrogen bonds with the acceptor groups of the RNA bases. Ring IV of neomycin is less well defined and protrudes around the flap base into the solvent.

Inhibition of catalytic RNAs by aminoglycosides

Catalytic RNAs or ribozymes are RNA molecules that can catalyse chemical reactions, such as the self-cleavage or religation of the phosphor–sugar backbone linkage. They are widely found in nature providing an indispensable biological activity. Thus, in the tRNA-processing enzyme ribonuclease P, the RNA moiety has a catalytic activity, which generates the mature 5′ terminus of an array of tRNA molecules. Self-splicing introns often occur within ribosomal genes, as in Tetrahymena rRNA. The extensive data on the inhibition of catalysis is condensed in Table 1.

Ribonuclease P RNA

A variety of aminoglycosides have been found to interact with the RNA subunit of the ribonuclease P ribonucleoprotein [30•]. Inhibition is found in the presence or absence of ribonuclease P protein, but only at low ionic strengths (typically, 1 mM NH₄, 10 mM spermidine and 10 mM MgCl₂, conditions in which neomycin B exhibits the highest cleavage inhibition
with an apparent inhibition constant \(K_i\) of 35 \(\mu\)M). Again, paromomycin is a less efficient inhibitor than neomycin with a \(K_i\) of around 190 \(\mu\)M. In line with the latter result, kanamycin A (position 1 = OH) is not inhibitory, whereas kanamycin B (position 1 = NH\(_2\)) is. The importance of the number of protonated amino groups is further supported by an increase of inhibition at lower pHs. Increasing the concentration of MgCl\(_2\) suppressed the inhibition caused by neomycin B. At elevated ion concentrations (100 mM NH\(_4\) and 100 mM MgCl\(_2\)) no cleavage inhibition is observed [30•]. An earlier study had found that the non-aminoglycoside puromycin is inhibiting under these conditions, whereas other antibiotics, including streptomycin, kasugamycin, sparsomycin and spectinomycin, had no inhibitory effect [31]. The lead(II)-induced cleavage patterns in the presence and absence of neomycin B are different and point to loop P15 as part of the

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**Figure 3**

Similarities between the (a) secondary and (b) tertiary structures of hairpins found in RNA aptamers raised against aminoglycosides and the fragment of 23S rRNA binding to protein L11. (a) Schematic representations of a conserved hexanucleotide loop of 23S rRNA (positions 1092–1099 in *E. coli*) [78–80] and of the aptamers binding to tobramycin [27,29••] and neomycin [29••], illustrating the similarities in the architectural folds (enclosed in the box). In each drawing, the arrow indicates the U-turn, typical of the anticodon structure in tRNAs, and the chevron indicates the bulging base flapping back onto the antibiotic. The nature of the non-canonical basepairs is indicated below, according to the following code: A, anti-anti G-A pair; B, bifurcated pair; S, sheared G-A pair; W, wobble-type pair. In the hexanucleotide of the 23S rRNA, the U-U contact is made by a single CH…O hydrogen bond between the O2 of the 5' uracil and C6 of the 3' uracil (distance 3.24 Å) [80].

**(b)** Stereo-views of the common regions of the structures boxed in (a). Top, the conserved hexanucleotide as extracted from the crystal structure of the complex between a 58 nucleotide RNA fragment and the protein L11 [80]; middle, the aptamer I of tobramycin [27]; bottom, the aptamer of neomycin B [29••]. The aminoglycoside is shown in white ball-and-stick representation. The arrowhead on the ribbon indicates the 3'-end. In the tobramycin aptamer, the U-U pair is the second basepair from the top, whereas in the neomycin aptamer, the G-A pair is third from the top. The van der Waals contacts between the bulging residues and the aminoglycosides are apparent (e.g., the syn conformation of the bulging guanine in the neomycin aptamer [29••]). The protruding guanine of the sheared G-A pair in the GNRA-like loop of the neomycin aptamer pushes the antibiotic upwards. Interestingly, NMR data [78,79] indicate that the U-U pair is often broken so that the 3'-uracil is bulging, as in the aptamer I of tobramycin. It is important to note that the conserved hexanucleotide loop is part of the GTPase center of the ribosome and belongs to the binding site of thiostrepton, a cyclic antibiotic of the thiazole family [81]. Therefore, it is tempting to suggest, by analogy with the structures of the aminoglycoside family, that thiostrepton would displace the equilibrium between the closed and open form of the U-U pair toward the open conformation, thereby disrupting the tertiary contacts formed by the loop in the GTPase center [80].
**Table 1**

Inhibition effects of various aminoglycosides on autocatalytic RNA cleavage*.

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*All concentrations are in µM unless otherwise stated. Superscripted letters refer to the references at the foot of the table. †References provide information on Kᵢ and Nᵢ where indicated. Kᵢ is the dissociation constant for the reversible dissociation reaction of an enzyme–inhibitor complex that forms before the irreversible inactivation step in a very dilute solution; Nᵢ represents the concentration of antibiotics required for 50% inhibition of the reaction.
aminoglycoside binding site. This conclusion is further supported by increases in $K_i$ upon mutations in the P15 loop.

**Inhibition of the self-splicing of the td group I intron**
The self-splicing of several group I introns (for a review, see [32]) is inhibited by a variety of aminoglycosides (reviewed in [6,8]). For the td intron (of the thymidylate synthase gene), footprinting experiments revealed two binding sites: one in the internal loop between stems P4 and P5 and the other in the catalytic core close to the guanosine-binding site [33•]. The inhibition is non-competitive with the guanosine cofactor and depends on pH and magnesium ion concentration. Changes in the patterns of hydroxyl radical cleavage induced by Fe$^{2+}$ (which substitute for Mg$^{2+}$) indicate that magnesium ions are replaced during binding of the aminoglycoside. The docking of neomycin to the core of the group I td intron in the three-dimensional model [34] showed that the protonated amino groups at positions 6' or 2' of ring I can displace one catalytic ion, whereas the amino group at position 1 of ring II might replace the second catalytic magnesium ion. The docking brings the 3-amino group of ring II in close proximity to the N3 position of the bulged C870 in stem P7 [33•]. The td intron is inhibited 300-times less by paromomycin than by neomycin. Interestingly, a cytosine–uracil mutation of the bulge in stem P7 renders the intron 500-fold more resistant to neomycin than the wild type (with an almost unchanged sensitivity towards paromomycin).

**In vivo** experiments indicate that neomycin B inhibits intron splicing indirectly by interfering with the translation step at the ribosomes [35•]. Interestingly, neomycin B also enhances mis-splicing in vivo because of the formation of an alternative hairpin structure containing a cryptic 3'-splice site (i.e. a site not normally used). The P4/P5 internal loop is one discriminating recognition site for the substrate helix containing the 5'-splice site and is one of the binding sites of neomycin B.

**Small catalytic RNAs**
RNA from the human pathogen hepatitis delta virus (HDV) and that from a number of small plant pathogenic viroids and virusoids, such as the *Tobacco ringspot virus* satellite RNAs, require RNA self-cleavage for the generation of the unit-sized RNA molecules following replication. The ‘hammerhead’ motif was identified on the positive (+) strand of the satellite RNA of the *Tobacco ringspot virus* RNA, whereas on the negative strand a different structural motif called a ‘hairpin’ was discovered. The structures of small ribozymes have been recently reviewed [36–39].

**Hammerhead ribozyme**
Among the several aminoglycosides tested, neomycin B is the strongest inhibitor of the autocatalytic activity of the hammerhead ribozyme with an $N_i$ (the concentration at which 50% of the ribozyme reaction is inhibited) of 1.5 μM [40]. Neomycin is in competition with magnesium ions displacing five divalent ions upon binding to the RNA [41]. The inhibition is independent of the protonation state of the amino group at position 3 of ring II, but inhibition is lowered when the amino groups with an intermediate $pK_a$ are deprotonated and inhibition decreases further above a pH of 8.0. Further experimental studies have demonstrated that the degree of inhibition correlates with the number of positive charges [42•] and with the basicity of the ammonium groups [43].

Molecular dynamics simulations showed that several solution conformations of neomycin B and tobramycin could be docked into the cleft of the hammerhead ribozyme [44•]. The intramolecular distances between the magnesium ion binding sites (around 4 Å, 8 Å and 11 Å) are complementary to the distances calculated between different amino groups of the aminoglycoside. Inhibition of the cleavage reaction proceeds by replacement of three to four magnesium ions found in the crystal structure around the cleavage site. Most of the contacts made by the amino groups are with the phosphate groups of the RNA backbone in the formally single-stranded regions with the exception of two direct hydrogen bonds to the N7 atom of the last adenine forming a Watson–Crick basepair in stem I as well as to the N4 base amino group of the following cytosine [44•]. The weaker inhibition of tobramycin compared to neomycin B [41,45] is reflected by the observation of fewer direct and water-mediated contacts with the RNA. A fluorescein-labelled hammerhead ribozyme study suggested that neomycin B binds as a dimer or binds to more than one site and induces conformational changes in the RNA [46]. A covalently linked neomycin dimer exhibited a stronger inhibition of self-cleavage compared to the unlinked molecule.

**Hairpin ribozyme**
A range of aminoglycosides are able to inhibit the cleavage reaction of the hairpin ribozyme in the presence of magnesium ions, but at least 10-fold less efficiently than for the hammerhead and HDV ribozymes [47••]. From the members of the neomycin family, neomycin B was the best inhibitor with an apparent $K_i$ of 190 μM. Substitution of amino groups or removal of ring system IV, or III and IV together, results in a loss of inhibition. In the tobramycin class, kanamycin B is the strongest inhibitor ($K_i$ of 400 μM), but the best inhibitor from any class in the case of the hairpin ribozyme is 5-epi-sisomycin, with a $K_i$ in the nanomolar range (0.6 μM). The inhibition is found to be 20-fold more effective at pH 6 than at pH 8.5. The addition of aminoglycosides does not interfere with the binding of the substrate strand, but instead acts by displacement of magnesium ions.
Surprisingly, aminoglycosides induce a site-specific cleavage in the absence of magnesium ions with rates only 13–20-fold lower than in their presence. The importance of the number of amino groups present and their protonation state is supported by the study of the pH-dependence of aminoglycoside-induced cleavage. By comparison, the hydrolysis rate of a dinucleotide (ApA) in the presence of a 30-fold higher concentration of neomycin (at 50°C and pH 8) is 9000-fold lower than cleavage in the presence of magnesium (about 100 times faster than the background rate [48]). Polyamines are also able to fold the hairpin ribozyme into its correct tertiary structure [49] and are catalytically active (in decreasing order with the number of amino groups: spermine > spermidine > NH4+) with single turnover catalytic rates barely 45-fold slower than with magnesium ions [47**,50]. Interestingly, polyamines enhance considerably the magnesium-dependent self-cleavage of the Neurospora VS ribozyme [51] and promote self-cleavage of the Coconut cadang cadang viroid [52]. It has been shown that diamines and oligoamines are able to catalyze RNA hydrolysis [48,53].

**Human HDV ribozyme**

Neomycin inhibits HDV self-cleavage with a $K_i$ of 28 µM and the inhibition can be reversed by increasing magnesium ion concentration in a competitive manner [54]. Paromomycin displays a similar footprint to neomycin, but no cleavage inhibition is observed. In vivo experiments of HDV RNA replication in cell line or transfected cells does not show inhibitory effects with the aminoglycosides neomycin B and tobramycin [55]. It is argued by the authors that the full-length viral RNA of HDV or its complexes with proteins (or maybe polyamines) may prevent aminoglycosides from interacting.

**Drug design**

Compared to natural sites, the binding affinities provided by in vitro selected aptamers are a factor of at least 1000 higher, most probably because of a reduction in the possibilities of docking to the binding site (despite an induced-fit recognition mechanism). This fact is used to control in vivo gene expression by aminoglycoside-binding aptamers attached to 5′-untranslated regions [56*].

To probe the specificity of neomycin B-RNA interaction, a series of synthetic analogs have been synthesised, testing the effect of positive charges and the idose sugar (ring IV). Whereas the number of positive amino groups increased the binding affinities, even for an acyclic side chain, the idose ring adds to the specificity of the binding towards the 16S rRNA [57]. Specific efforts were made to increase the binding affinity and specificity by adding various compounds: positively charged fluorescent dyes [58–60]; RNA intercalators [61]; dimeric aminoglycosides [62,63]; or additional amino groups [42*,63]. Chemically synthesised 1.3-(2) aminol-containing molecules are found to compete with aminoglycoside–RNA interactions [64]. Novel small molecular RNA binders might be discovered in the future by the use of fast screening methods [65–67].

In the search for novel antiretroviral ligands, new peptidomimetic substances capable of inhibiting viral transcription by blocking the interaction between the Tat protein and its binding site on the HIV-1 RNA (TAR sequence) have been built [68]. This interaction is mediated by a basic region of the protein containing six close arginine residues. Furthermore, an artificial peptide with only one arginine is sufficient to bind to the TAR with the same affinity [69]. Some chimeras possessing the sugar skeleton of the aminoglycosides with guanidinium groups at variable positions bind the RNA with a $K_d$ of 20–400 nM. In addition, the results showing that 1 mM of any derivative is not cytotoxic give a positive impulse to the design of efficient targeting molecules.
A strategy for docking cationic drugs to an RNA target based on Brownian and molecular dynamics simulations has been proposed [16•]. The strategy (Figure 4) partitions hierarchically the interactions between the rather unspecific electrostatic and the highly shape-sensitive van der Waals contacts. Firstly, for a given three-dimensional RNA fold, the electronegative pockets created by the fold are predicted by Brownian dynamics simulations of cation diffusion [70]. Several starting models are then built by anchoring the positively charged groups of the drug into the pockets of electronegative density. Secondly, molecular dynamics calculations allow the evaluation of shape-sensitive interactions such as van der Waals’ forces and hydrogen-bonding contacts. The strategy has successfully modelled known RNA–aminoglycoside complexes and allowed the deduction of TAR–aminoglycoside models. Another method [71] relies on three-dimensional structure/activity relationship computations with a docking protocol in which extensive searching of ligand conformational space is coupled with molecular dynamics simulations for optimization. The method has been applied to deduce a model for the rev-response element-RNA interaction.

Progress in techniques
Fluorescently labelled RNA molecules [46] or aminoglycosides [66,72•] can now be used for spectroscopic analyses by polarisation or anisotropy [20,61,64,66,72•] and fluorescence intensity [46,58,59]; the techniques of lifetime measurements, steady-state and stop-flow kinetics or fluorescence resonance energy transfer (FRET) should come in the future. Fluorescent-dyelabelling also allows high-throughput screening of aminoglycoside or RNA libraries [66]. Electron spray ionisation–mass spectrometry (ESI-MS) has been used to measure the stoichiometry between small molecules and RNA or RNA–protein complexes [22,73].* In vitro biosensor [74] or surface plasmon resonance [17•,57,75] techniques are used for measuring real-time kinetics of RNA binding. These biophysical techniques facilitate the study of interactions of aminoglycosides to specific sites and the conformational changes induced upon binding. A tobramycin–EDTA construct has been used for detecting the binding sites of drugs and for chemical probing upon RNA interaction [45].

Conclusions
Aminoglycoside antibiotics interact with a great variety of RNA molecules and any biological function involving RNA is a potential target. Because RNA molecules are highly charged, metal ions participate in RNA three-dimensional folding and provide active centers in catalytic RNA molecules. The interactions between aminoglycosides and RNA are dominated by the number and basicity of amino groups in the aminoglycoside [42•,76]. Experimental and theoretical data show that structural complementarity can explain specific binding and catalysis inhibition induced by aminoglycosides: positively charged ammonium groups match the negatively charged metal-ion-binding pockets created by the electrostatic field, which is generated by the RNA fold, displacing ions [44•,76]. These can be structural or catalytic divalent metal ions. Therefore, aminoglycosides can promote conformational changes, prevent folding of the RNA into an active tertiary architecture or displace catalytically active ions. Polyamines, like magnesium ions, promote RNA folding and rescue destabilizing mutations. It is now clear that polyamines can also induce RNA catalysis. Thus, in vitro aminoglycosides (or designed drugs) will be confronted by many competitors including proteins, metal ions and polyamines, which might complicate straightforward transfer from the in vitro models.

An analysis of the dynamics at an RNA–protein interface led to the conclusion that interface inhibitors should contain flexible cationic groups for competition with other charges and shield the polar contacts made to the RNA from the environment [77]. Indeed, NMR studies of RNA aptamers showed that aminoglycosides bind in opened and widened deep grooves of RNA helices at the interface between non-Watson–Crick basepaired regions and hairpin loops with single base bulges serving as spacers or participating actively by encapsulating the interacting molecule. The aminoglycosides fill the available space and make several specific contacts to the RNA bases and backbone. NMR studies provided the first views into the principles and patterns of aminoglycoside recognition and discrimination at the atomic level. The solution structures open the way for rational drug design using theoretical approaches and combinatorial chemistry to improve the binding specificity and affinity of antibiotics.

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References and recommended reading
Papers of particular interest, published within the annual period of review, have been highlighted as: • of special interest
•• of outstanding interest


