Thomas Hermann
Eric Westhof
Institut de Biologie Moléculaire et Cellulaire du CNRS, UPR 9002, Equipe de Modélisation et Simulation des Acides Nucléiques, 15 rue René Descartes, F-67084 Strasbourg, France

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Saccharide–RNA Recognition

Abstract: *Among saccharides, the antibiotics of the aminoglycoside family are the best-studied class of molecules interacting with RNA. By binding to RNA targets, aminoglycosides act as inhibitors of protein biosynthesis, they interfere with protein–RNA interaction of retroviral regulatory elements, and they inhibit the catalytic action of ribozymes. Here, we survey the available data on molecular structural details of aminoglycoside–RNA interaction.* © 1999 John Wiley & Sons, Inc. Biopoly 48: 155–165, 1998

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INTRODUCTION

Saccharides are multifunctional molecules with polar substituents suitable for the interaction with polyanionic RNA targets. While saccharides are widely found in nature, both in free form and as glycosyl substituents of other biomolecules such as proteins, their roles in interactions with nucleic acids are greatly unknown. A well-explored exception on the *terra incognita* of saccharide–nucleic acid interaction are the aminoglycosides (Figure 1), which have been known for a long time as potent antibiotic therapeutics against bacterial infections.[1] Aminoglycosides bind specifically to the RNA component of bacterial ribosomes, leading to miscoding during translation and ultimately to bacterial cell death.[2] Other RNA molecules have since been identified as specific targets for aminoglycoside binding,[3,4] among them catalytic RNAs (ribozymes)[5–7] and, importantly, two regulatory RNA elements, the trans-activating response element (TAR) and Rev-response element (RRE) of the human immunodeficiency virus (HIV).[8,9] Binding of aminoglycosides to the RNAs inhibits catalytic activity of the group I intron, hammerhead, and hepatitis delta virus (HDV) ribozymes, and prevents binding of the cognate viral proteins Tat and Rev to TAR and RRE.

Because of their importance as therapeutics and their diverse biological functions, aminoglycosides constitute a paradigm in the study of RNA recognition by drugs. At physiological pH, most of the amino groups in aminoglycosides are protonated.[10] Unlike many other cationic molecules that bind to RNA, aminoglycosides are able to discriminate between different three-dimensional motifs within large RNA

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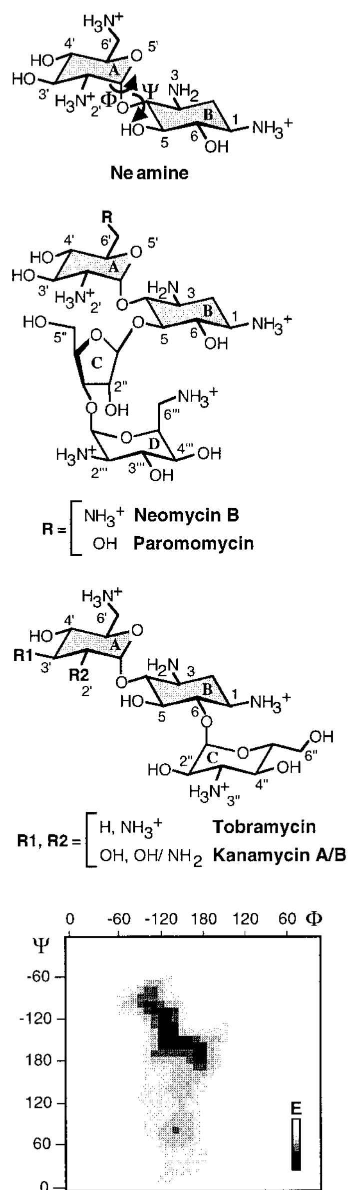

FIGURE 1 Structures of aminoglycosides that bind to RNA. Neamine, indicated by grey shading, is the common core moiety of all aminoglycoside antibiotics. Neomycin and paromomycin belong to the 5-substituted class of neamines; tobramycin and the kanamycins are 6-substituted derivatives. At the bottom, an energy map for rotations about the Φ and Ψ torsion angles between rings A and B of neamine is displayed. Low energy conformers of neamine are indicated by dark shading. The map was calculated using a molecular mechanics force field.[17]

folds. Information on structural details of aminoglycoside–RNA recognition has been emerging only very recently when three-dimensional models for aminoglycoside–RNA complexes derived by nmr[11–14] and molecular modeling[15–17] became available. In this review, we will focus on aminoglycoside–RNA interaction as an example of saccharide–RNA recognition, given both its importance for drug development and the large quantity of available data.

RIBOSOMAL RNA

Aminoglycosides exert their antibiotic activity by binding to the A site within the decoding region of bacterial 16S ribosomal RNA (rRNA), thereby interfering with accurate protein synthesis. The interaction of aminoglycosides with either full-size 16S rRNA or oligonucleotides derived from the A site has been studied using chemical footprinting,[18–23] modification interference,[24] in vitro selection,[25] fluorescence measurements,[26] surface plasmon resonance,[27,28] nmr,[12,14,29] and molecular simulations.[17] Detailed three-dimensional information on aminoglycoside recognition by ribosomal RNA is provided by the nmr structure of a complex between the neomycin-class compound paromomycin and an A-site oligonucleotide (Figure 2).[12,14,29]

The nmr studies have revealed the chemical groups of the conserved nucleotides required for the specific binding of neomycin-class aminoglycosides to the bacterial 16S rRNA A site (Figure 3).[12,14] The RNA makes contacts to the ammonium and hydroxyl groups of paromomycin via the N7 atoms of several adenines and guanines, the N1 atoms of two adenines, the carbonyl O4 atom of U1495, as well as several phosphate groups. Most of the base contacts are to universally conserved nucleotides; however, there are distinct interactions with bases at positions 1491 and 1408, both of which display different conservation patterns in eucaryotes and eubacteria (see below). In paromomycin, hydroxyl groups are involved in contacts to phosphate groups, while ammonium groups in the conserved neamine moiety interact with both phosphates and bases. Ring A of the neamine moiety stacks below the Watson–Crick-paired base of G1491 that, together with the A1408–A1493 pair, forms a tight pocket for the carbohydrate ring (Figure 2). In 16S rRNA of both eucaryotes and some antibiotic-resistant eubacteria, the nucleotide at position 1491 is not base paired and A1408 is changed to a G, abolishing the aminoglycoside-binding capacity of the RNA.[12,30] The structural changes caused by these sequence differences in ribosomal RNA are responsible for the relatively low toxicity of aminoglycosides in eucaryotic organisms, including humans. Conversely, a genetic predisposition, based on an A to G point mutation in mitochondrial 12S rRNA at the position corresponding to nucleotide 1491 in 16S

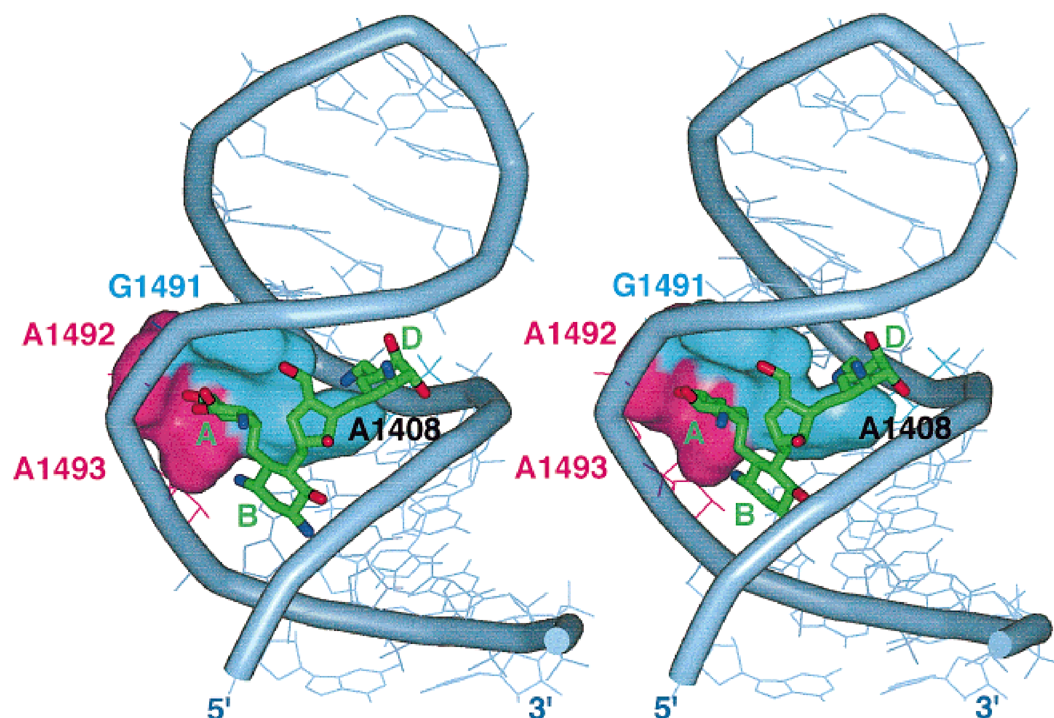

FIGURE 2 Stereo view of the three-dimensional nmr structure of a complex between paromomycin (green stick model) and an oligonucleotide representing the 16S rRNA A site of *Escherichia coli* (blue wire and backbone tube).[12] Hydroxyl and ammonium groups in the aminoglycoside are colored red and blue, respectively. The bases of A1408, G1491 (both in cyan), A1492, and A1493 (both in magenta), forming a tight pocket around ring A of paromomycin, are shown in space-filling surface representation. Binding of the drug displaces A1492 and A1493 out of the deep groove. Sequence differences between eucaryotes and eubacteria at positions 1408 and 1491 are responsible for the low affinity of aminoglycosides to eucaryal 16S rRNA.[12,30]

rRNA, leads to increased toxicity of aminoglycosides ultimately causing drug-induced deafness in humans.[31,32]

While rings A and B of paramomycin make highly specific contacts to the RNA in the set of nmr structures, ring D is partially disordered. Indeed, rings C and D contribute only weakly to specific aminoglycoside binding.[12,33] Electrostatic calculations have revealed that rings C and D are located within regions of high negative charge density in the electrostatic field created by the RNA fold.[17] Taken together, these findings suggest that rings C and D are involved in shape-insensitive electrostatic interactions so that several conformers of the aminoglycoside can be accommodated. A similar plasticity of electrostatic RNA–aminoglycoside interactions was proposed for the hammerhead ribozyme[15] and TAR RNA (see below).[17]

Major differences in both specificity and strength of binding between neomycin-class and kanamycin-like compounds (Figure 1) have been revealed by quantitative surface plasmon resonance studies of

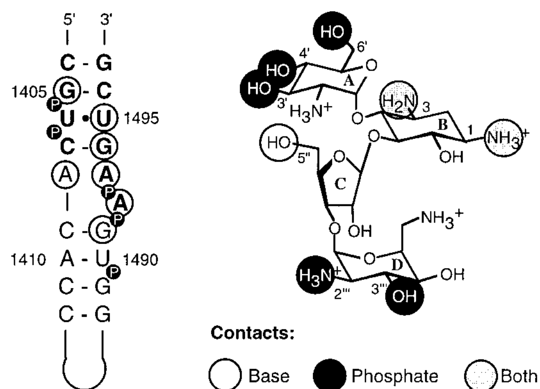

FIGURE 3 Summary of interactions between paromomycin and the 16S rRNA A site determined by nmr and chemical modification interference.[12,24] Left panel: Nucleotides that form contacts to the aminoglycoside via their bases are encircled in the secondary structure representation; interacting phosphates are shown explicitly. Conserved nucleotides are in bold. Right panel: In the aminoglycoside, substituents are marked differently depending on their interaction counterparts in RNA.

aminoglycoside binding to the A site of 16S rRNA.[28] The 4,5-linked aminoglycosides, such as neomycin B and paromomycin, bind to 16S rRNA stronger and with higher specificity compared to the 4,6-linked kanamycin derivatives. Based on this data and on electrostatic calculations, it has been suggested that several conformers of kanamycin-class compounds can bind to 16S rRNA in different orientations[17] while neomycin-like aminoglycosides occupy a single defined orientation with respect to their neamine core. In the paromomycin/A site complex, rings A and B of the aminoglycoside are oriented in such a way that the bases of A1492 and A1493 are displaced out of the deep groove, leading to a local conformational change in the RNA. This structural change has been suggested to be at the origin of the deleterious effect of aminoglycosides on the translation process.[29] Kanamycin-class compounds that can be accommodated in various binding orientations might thus be less efficient in inducing the conformational change. The stronger directionality of the neomycin-class antibiotics binding to the A site is based on electrostatic interactions of rings C and D, which fit inside a negatively charged cavity in the deep groove of the RNA.[17] The importance of rings C and D for conferring directionality to aminoglycoside binding to the A site is underscored by nmr studies showing that unsubstituted neamine, lacking additional rings C and D, binds to the A site in alternate orientations.[14]

CATALYTIC RNAS

Ribozymes are RNA molecules that catalyze chemical reactions in the absence of proteins. All known naturally occurring ribozymes catalyze phosphodiester bond cleavage or transesterification. Among these ribozymes, the group I introns,[5,34,35] the hammerhead motif in the RNA genomes of plant viroids,[6,36,37] and the ribozymes from HDV[7,38] are inhibited by aminoglycoside antibiotics. While these three classes of ribozymes depend on divalent cations for catalysis, the hairpin ribozyme, for which metal ions are dispensable for self-cleavage,[39,40] is not inhibited by aminoglycosides.[35]

The hammerhead ribozyme is a small catalytic three-way junction RNA that cleaves a phosphodiester bond within its own backbone in a metal ion-catalyzed process.[41] Inhibition of the hammerhead ribozyme by aminoglycosides provides a simple paradigm system for the study of drug–RNA interaction.[42] Aminoglycosides from both classes of 5- and 6-substituted neamines and also neamine itself inhibit the hammerhead catalytic reaction by binding to the enzyme–substrate complex.[6] Three lines of experimental evidence suggest that electrostatic interactions between the RNA and the positively charged drug play a major role in the inhibition of hammerhead cleavage by aminoglycosides. First, it has been shown that neomycin acts on the ribozyme by competing for binding to the RNA with about five Mg^{2+} ions.[36] Second, increasing the number of positively charged ammonium groups in aminoglycosides leads to higher binding affinities of these synthetic compounds.[43] Third, enhancing the basicity of ammonium groups by removing neighboring electron-withdrawing hydroxyl groups increases binding affinities of aminoglycosides.[37]

While these findings clearly support the importance of electrostatic interactions for aminoglycoside binding to the hammerhead RNA, the mere presence of positively charged groups is not sufficient for creating a hammerhead inhibitor since other polycations, such as the polyamine spermine, do not inhibit hammerhead catalysis.[44] The specific binding of aminoglycosides to the hammerhead ribozyme can be explained by a structural electrostatic complementarity between the array of positively charged ammonium groups in the antibiotics and the negatively charged metal ion binding sites in the RNA.[15,42] Molecular dynamics simulations have revealed that low-energy solution conformers of aminoglycosides (Figure 1, bottom panel) provide a defined set of interammonium distances matching the sets of intermagnesium distances in the crystal structure of the hammerhead ribozyme.[45] Several solution conformers of neomycin and tobramycin have been successfully docked to the hammerhead RNA by using the crystallographic Mg^{2+} sites for positioning the ammonium groups of the aminoglycosides (Figure 4). By complementing in space the electrostatic potential created by the three-dimensional fold of the hammerhead RNA, the aminoglycosides could displace simultaneously several metal ions required for catalysis.[44]

Details of aminoglycoside–RNA recognition have been revealed by molecular dynamics simulations of docked antibiotics/hammerhead complexes in solution.[15] Contacts occur between polar substituents in the aminoglycosides and both phosphates and bases in proximity of the cleavable phosphate in the RNA (Figure 5). The most important contributions to the aminoglycoside–hammerhead interactions in all docked complexes involve the ammonium groups of the neamine core. These specific contacts are accompanied by varying nonspecific and water-mediated interactions. The accommodation of different solution conformers of aminoglycosides by the hammerhead RNA is facilitated by the plasticity of solvent-medi-

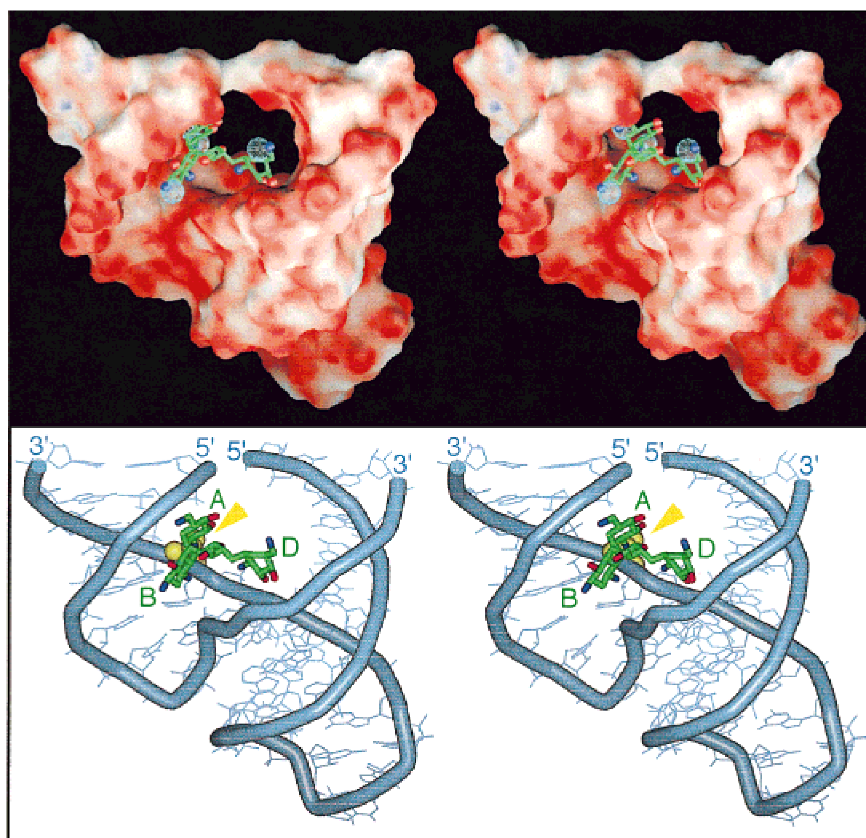

FIGURE 4 Stereo view of a representative modeled complex of neomycin B bound to the hammerhead ribozyme.[15] The model was constructed by docking a solution conformer of neomycin to the crystal structure of the hammerhead RNA.[45] The top panel displays the electrostatic complementarity between the metal ion binding sites in the electronegative cavity of the hammerhead RNA and the positively charged ammonium groups in the aminoglycoside. Neomycin is shown in stick representation with ammonium groups in dark blue and hydroxyl groups in red. The Mg^{2+} ions in the crystal structure of the ribozyme are overlaid as light blue spheres. The electrostatic potential around the RNA is projected on the molecular surface with negatively charged patches indicated in red. The bottom panel shows the RNA in stick and backbone tube representation. The cleavable phosphate is marked in yellow. GRASP[80] was used for preparing the top panel.

ated contacts. Rearrangement of water molecules at the interface between the bound drug and the RNA provides a maximum number of hydrogen bonds.

The hypothesis of electrostatic complementarity derived for the hammerhead RNA may also explain the aminoglycoside inhibition of the two other classes of metal-dependent ribozymes, namely the HDV ribozymes and the group I introns.[15,35] For both ribozymes, inhibition of catalysis by aminoglycosides is strongly dependent on pH and Mg^{2+} concentration.[7,35] A direct competition between neomycin and Mg^{2+} ions has been demonstrated for the HDV ribozyme.[7] An analysis for complementarity between ammonium groups in aminoglycosides and metal ion binding sites in the HDV ribozyme has not yet been performed because its three-dimensional structure, which became available only very recently,[46] does not reveal the positions of metal ions in proximity of the catalytic center.

For group I introns, the displacement of metal ions, essential for self-splicing, by neomycin B has been recently demonstrated experimentally.[35] Self-splicing of group I introns is a two-step process that requires at least two divalent metal ions[47,48] and guanosine as a cofactor.[49] Aminoglycosides inhibit catalysis of group I introns noncompetitively with respect to binding the G cofactor, indicating that these antibiotics do not interfere with the G-binding site.[5,34] Based on footprinting experiments using Fe^{2+}-generated hydroxyl radicals on complexes of neomycin and the *td* group I intron, the position of the aminoglycoside has been determined[35] in a three-dimensional model of the

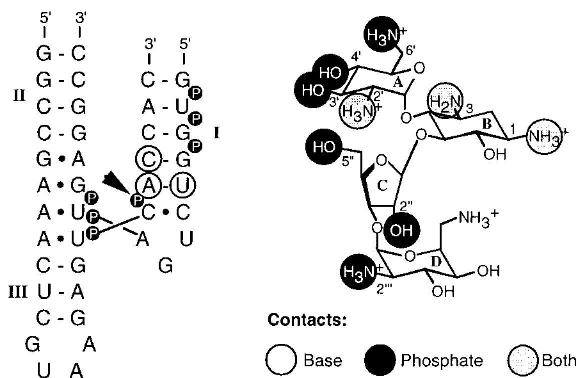

FIGURE 5 Summary of interactions between neomycin B and the hammerhead ribozyme observed in molecular dynamics simulations of several docked complexes.[15] Left panel: The secondary structure of the hammerhead RNA is drawn to resemble the three-dimensional folding in the crystal structure (see Figure 4). Nucleotides that form contacts to the aminoglycoside via their bases are encircled; interacting phosphates are shown explicitly. The cleavable phosphate is indicated by an arrow. Right panel: In neomycin, substituents are marked differently depending on their interaction counterparts in RNA.

RNA.[50,51] In line with previous suggestions,[52] at least two binding sites for neomycin B have been detected on the *td* intron.[35] One of the sites comprises residues of the catalytic center where, according to a previously derived model,[47] two divalent metals are bound. The docking to the *td* intron of neomycin solution conformers, as determined by molecular dynamics simulations,[15] was performed,[35] following the strategy developed for the hammerhead ribozyme,[15] which assumes electrostatic complementarity between the RNA-bound metal ions and ammonium groups of the aminoglycoside. In the resulting complexes, different solution conformers of neomycin could displace both catalytic metal ions by binding ammonium groups of the neamine core into cation binding sites. In line with the experimental findings, the G-cofactor binding site is not sterically hindered by neomycin docked at positions that were proposed on the basis of electrostatic complementarity.[35]

HIV REGULATORY RNA ELEMENTS

The specific binding of aminoglycosides to RNA can inhibit protein–RNA interactions by both direct competition for a binding site and noncompetitive mechanisms. Two RNA motifs in the genome of HIV, namely TAR and RRE, are targets for aminoglycosides.[8,9] The interactions of TAR and RRE with their cognate viral regulator proteins Tat and Rev are inhibited by aminoglycosides, which thus interfere with the transcription (TAR–Tat) and the nuclear transport (RRE–Rev) of viral mRNA.[53]

The RRE, a stretch of 234 nucleotides within the viral *env* coding region, is the binding site for the Rev protein that recognizes a bulge structure in the core of RRE.[54] The central element in RRE–Rev interaction is a noncanonical G · G base pair that leads to widening of the deep groove in the bulge region.[55,56] Chemical footprinting and mutation studies have revealed that neomycin B competes directly with the Rev protein for RRE binding by specifically recognizing nucleotides of the Rev-binding site, among them the G · G base pair.[8,55] Based on these experimental findings, three-dimensional models of RRE–aminoglycoside complexes have been constructed by docking neomycin B and tobramycin to a previously modeled conformation of the RRE core region.[16] The modeled complexes suggest a number of RNA–aminoglycoside contacts supported by experimental data (Figure 6). In the models, specific contacts are observed between amino groups of the neamine moiety and bases of the RNA, mostly guanines interacting with their Hogsteen face (O6 and N7 atoms).[16] The important contribution of the neamine moiety for the specific binding of aminoglycosides to RRE, suggested by the models, is in line with experiments

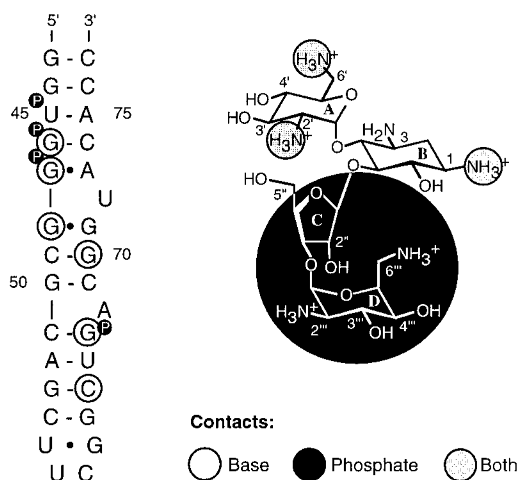

FIGURE 6 Summary of interactions between neomycin B and the RRE RNA deduced by static analysis of docked complexes.[16] Left panel: Nucleotides that form contacts to the aminoglycoside via their bases are encircled; interacting phosphates are shown explicitly. The cleavable phosphate is indicated by an arrow. Right panel: In neomycin, substituents are marked differently depending on their interaction counterparts in RNA. Substituents of rings C and D form exclusively electrostatic contacts with varying contributions, not given in detail in the literature.[16]

demonstrating that the neamine core is necessary for RRE–Rev inhibition.[8] Rings C and D of neomycin exclusively form contacts to the phosphate backbone, resembling their role in electrostatic interactions in the A-site/paromomycin complex (see above).[16]

In contrast to the competitive inhibition of RRE–Rev binding by aminoglycosides, the interaction between TAR and Tat is impeded noncompetitively by neomycin B.[57] The TAR motif forms a hairpin structure at the 5′ end of nascent viral mRNA transcripts.[53] A base triplet and a bulged nucleotide lead to a widened deep groove that is the target for the binding of the Tat protein in the three-dimensional structure of TAR.[58] Widening of the deep groove, albeit by a purine · purine base pair, has been already described as a characteristic structural feature of the Rev-binding site in RRE.

The noncompetitive inhibition of the TAR–Tat interaction by aminoglycosides suggests that the binding sites for the protein and the drugs on TAR do not essentially overlap. The residues necessary for Tat recognition have been determined by nmr studies on TAR–Tat complexes.[58–60] Two independent lines of evidence support the hypothesis that the binding of Tat and aminoglycosides involve largely disparate sets of contact sites in TAR. First, enzymatic footprinting experiments have revealed[61] that the bound neomycin protects nucleotides of the stem around C19 while the Tat protein binds to the bulge region (Figure 7).[58] Mutations at the bulge domain of TAR had little or no effect on the extent of neomycin binding.[57] Second, molecular dynamics simulations of docked neomycin–TAR complexes have shown[17] that the chemical groups of TAR interacting with the bound aminoglycoside are mostly located outside the bulge region (Figures 7 and 8). The three-dimensional models of neomycin–TAR complexes have been obtained by docking solution conformations of the aminoglycoside to the free TAR RNA guided by the electrostatic complementarity between negatively charged pockets in the RNA and the positively charged ammonium groups of neomycin.[17] In the molecular dynamics simulations of the docked complexes, base-specific interactions were recurrently observed between nucleotides of the stem regions of TAR and the amino groups of rings A and B of neomycin (Figure 7). Rings C and D contributed in varying electrostatic contacts with the phosphate backbone of the RNA. The differences between the neamine core forming specific interactions and the rings C and D participating in fluctuating electrostatic contacts have also been observed for aminoglycoside complexes of RRE and the 16S rRNA A site (see above).

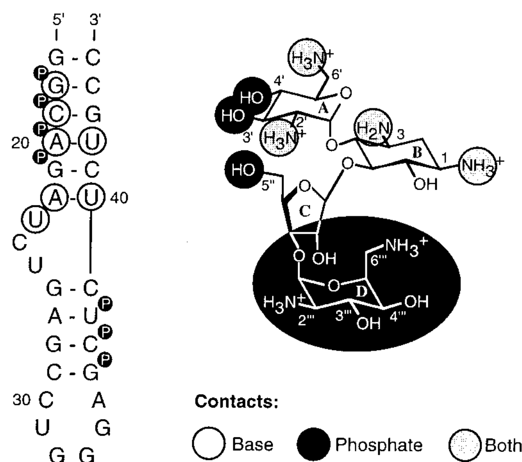

FIGURE 7 Summary of interactions between neomycin B and the TAR RNA recurrently observed in MD simulations of different TAR–neomycin B complexes.[17] Left panel: Nucleotides that form contacts to the aminoglycoside via their bases are encircled; interacting phosphates are shown explicitly. Right panel: In neomycin, substituents are marked differently depending on their interaction counterparts in RNA. Between ring D and phosphate groups, exclusively electrostatic interactions were observed dependent on the orientation of neomycin in the particular TAR–drug complex.

The binding region of neomycin on TAR, revealed by footprinting and simulation studies, supports the noncompetitive action of aminoglycosides in inhibiting TAR–Tat interaction. It has been suggested that the drugs might lock TAR in a conformation that is impeded for Tat binding.[17,58] The conformational change of TAR,[58–60,62] occurring after the protein has formed initial contacts in the widened deep groove,[63,64] might be impeded in the presence of the drugs.[17,61]

APTAMERS

RNA molecules, obtained by in vitro selection (SELEX) of pools of random sequences, which bind a substrate molecule with both high affinity and specificity, are called aptamers.[65] Aptamer RNAs recognizing saccharides have been synthesized for various aminoglycosides such as kanamycin A,[66] tobramycin,[67–69] neomycin B,[70] streptomycin,[71] and recently, for the monosaccharide galactose.[72] Here, we will only briefly outline some principles of saccharide–RNA recognition in RNA aptamer complexes since they are comprehensively described in the review by Dinshaw Patel in the present issue.

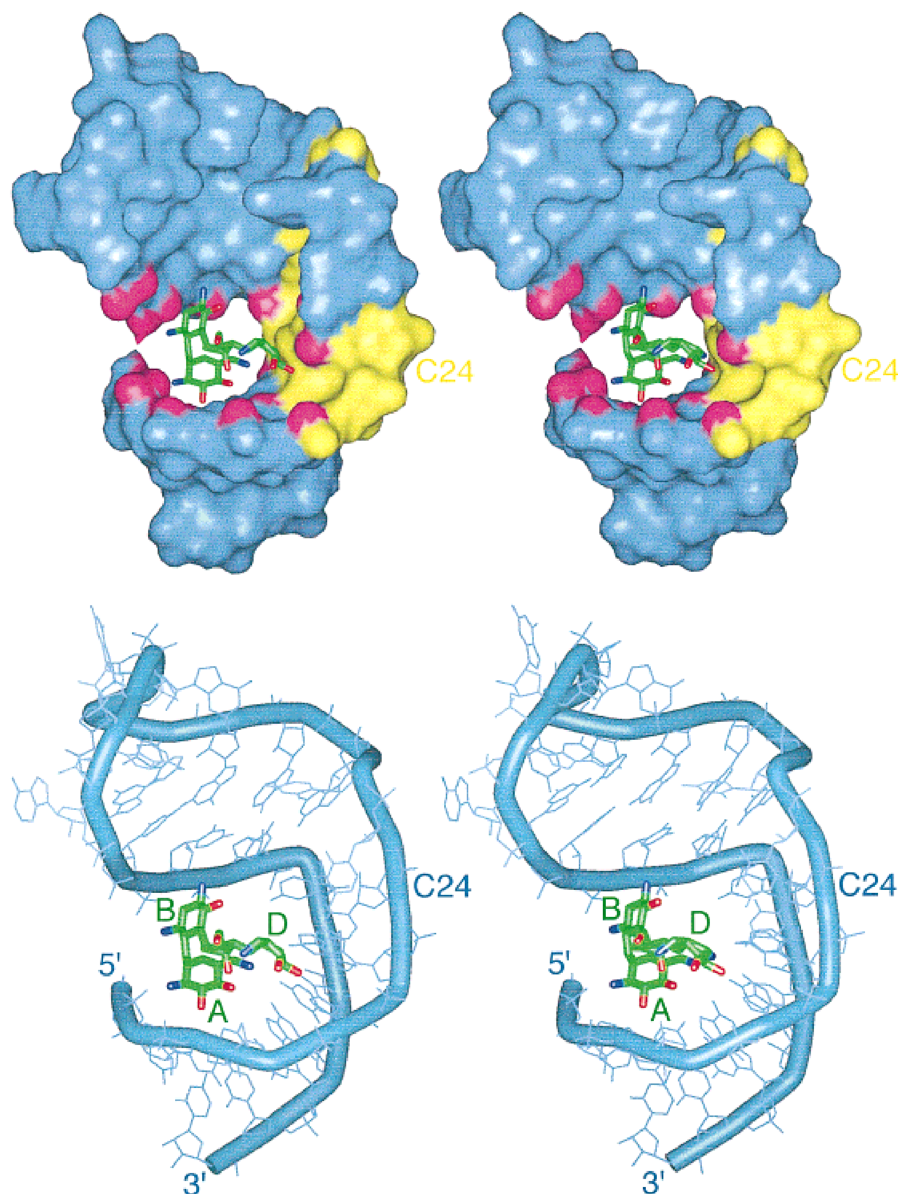

FIGURE 8 Stereo view of a representative modeled complex of neomycin B bound to the TAR RNA.[17] The model was constructed by docking a solution conformer of neomycin to predicted electronegative pockets in the nmr structure of free TAR.[62] Top panel: In the surface representation of the RNA, atoms recurrently found forming contacts with the aminoglycoside in molecular dynamics simulations are in pink, nucleotides involved in recognition of the Tat protein are in yellow. Neomycin is shown in stick representation with ammonium groups in dark blue and hydroxyl groups in red. Bottom panel: RNA in stick and backbone tube representation.

As a common feature, aminoglycoside apatmers bind the substrate in bulge or loop regions of the secondary structure.[11,13,70,73] Molecular details of aminoglycoside–RNA interaction have been revealed by the three-dimensional solution structures of two tobramycin–RNA aptamer complexes determined by nmr spectroscopy.[11,13] In both complexes, the aminoglycoside is bound in the widened deep groove of an RNA stem–loop structure tightly encapsulating the substrate. Noncanonical base pairs are extensively used for sculpting the aminoglycoside binding pocket in the loop region of the RNAs. The antibiotics are anchored to the RNAs by interactions between ammonium groups and both phosphates and bases.

However, the details of molecular recognition may differ between the aminoglycoside complexes of nat-

ural RNAs described above and the tobramycin aptamers. The specific interaction of aminoglycosides with the A site, the ribozymes, and the HIV regulatory RNAs requires the presence of the neamine moiety that plays a less important role in tobramycin–aptamer recognition. In the two aptamers, ring A of the neamine core is pointing away from the RNA into the solvent because this ring was linked to a solid support during the in vitro selection procedure. Thus, rings B and C of tobramycin are essentially contributing to the specific interaction between the aminoglycoside and the aptamer RNAs.

Importantly, it has been demonstrated recently that the highly specific aminoglycoside–aptamer interactions observed in vitro are functional also in vivo and can be used for controlling gene expression in living cells.[74]

CONCLUSIONS AND PERSPECTIVE

Aminoglycosides are saccharide derivatives highly specialized for recognition of RNA targets. The oligosaccharide backbone of aminoglycosides provides a three-dimensional scaffold for the arrangement of positively charged ammonium groups readily able to complement in space negatively charged pockets in the electrostatic field created by RNA folds. Shape-sensitive contacts between the RNA and conformationally restricted systems of rings, such as the neamine core in aminoglycosides, are accompanied by variable electrostatic interactions of more flexible extensions of the neamine core such as ring D in the neomycin-class antibiotics. Bridging water molecules play an important role in providing plasticity in these electrostatic interactions.

While our knowledge of saccharide–RNA recognition is restricted almost exclusively to the binding of aminoglycosides, it is tempting to speculate about the role of saccharide–RNA interactions for the recognition of RNA-binding glycoproteins. Data on glycosylated RNA-binding proteins is still extremely scarce.[75] However, since evidence is growing that glycoproteins are localized not only at the surface and lumenal compartments of cells but also exist in the nucleus and cytosol,[76] there is a good chance to find, among these "soluble" glycoproteins, RNA-binding factors in which the glycosyl substituents may participate in RNA recognition.

RNA recognition by oligosaccharides might be even more widely found in nature than the example of aminoglycosides suggests. Interestingly, both oligonucleotides and oligosaccharides are recognized by a common structural protein motif, the OB-fold,[77] emphasizing the inherent chemical similarity between the sugar–phosphate backbone of RNA and saccharide ring systems. Oligosaccharides could thus participate in the folding process of the three-dimensional structure of natural RNAs. This has been observed for artificial aptamer RNAs that do not possess a defined structure by themselves and do acquire a tight three-dimensional fold only upon induced-fit binding of their cognate substrate.[78] It is conceivable that such adaptive binding processes might also play a role for natural RNAs. Along this line, Davies and co-workers have suggested earlier[79] that the biological activity of some RNAs might depend on the binding of cofactors such as oligosaccharides acting as inducers and regulators.

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