Deciphering RNA recognition: aminoglycoside binding to the hammerhead ribozyme

Yitzhak Tor,[1] Thomas Hermann[2] and Eric Westhof[2]

Aminoglycoside antibiotics inhibit protein biosynthesis and various ribozymes. Structural electrostatic complementarity can explain the inhibition mechanism of the hammerhead ribozyme: positively charged ammonium groups match the negatively charged metal-ion-binding pockets created by the RNA fold's electrostatic field.

Addresses: [1]Department of Chemistry and Biochemistry, University of California, San Diego, La Jolla, CA 92093-0358, USA. [2]Institut de Biologie Moléculaire et Cellulaire du CNRS, 15 rue René Descartes, F-67084 Strasbourg, France.

Correspondence: Yitzhak Tor and Eric Westhof
E-mail: ytor@ucsd.edu
westhof@ibmc.u-strasbg.fr

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RNA molecules have key roles in many essential biological processes, including protein synthesis, transcriptional regulation, mRNA splicing and retroviral replication. The structural diversity of RNA, together with the lack of known RNA repair mechanisms, make RNA a challenging and important target for therapeutic intervention [1]. Our understanding of the modes by which RNA is recognized by ligands is far from being comprehensive, although significant advances have been made [2]. In order to target pivotal bacterial or viral RNA sites, one would like to be able to identify well-defined binding motifs that will facilitate the design and synthesis of new binders [3].

The functional diversity of RNA is based on the complex three-dimensional folds it can adopt. This structural sophistication leads to the formation of potential binding pockets for ions, ligands and proteins. The interaction of ligands with RNA is likely to be 'shape-specific' rather than sequence-specific. This adds to the complexity of the problem of designing effectors targeted at RNA, as the prediction of both the folded structure and putative binding sites for certain ligands is not at all trivial [4,5].

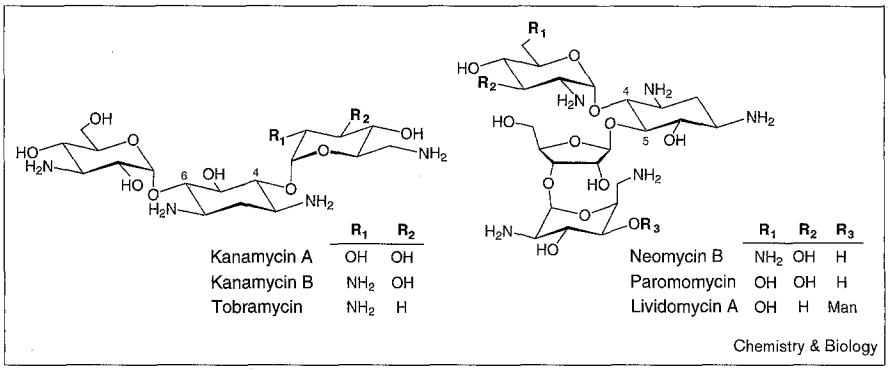
The discovery that aminoglycoside antibiotics bind to diverse RNA molecules such as ribosomal RNA [6], group I introns [7,8] and the hammerhead [9] and hepatitis delta virus [10] ribozymes, as well as the finding that antibiotics block the binding of the Rev protein [11] and Tat peptide [12] to their viral RNA targets, have attracted considerable interest [13,14]. Although at first glance it appears that these antibiotics interact with seemingly unrelated RNA molecules, they are likely to recognize similar structural motifs within RNA folds. Our understanding of aminoglycoside binding to RNA has advanced considerably recently. *In vitro* selection studies have identified various RNA sequences that bind aminoglycosides with high affinity [15–17], although these aptamers might not share the same binding characteristics as naturally occurring RNAs. Structures solved using nuclear magnetic resonance (NMR) have begun to reveal details of RNA recognition at the molecular level [18,19], and a number of other approaches have focused on issues of specificity in RNA–aminoglycoside binding [20–23].

The purpose of this article is to combine recent experimental and theoretical studies that have focused on the interactions between aminoglycoside antibiotics and the hammerhead ribozyme. We view this system as a paradigm that allows one to explore the interaction between small organic molecules and a catalytically active RNA. Although this system might not necessarily reflect the situation *in vivo*, it encompasses several valuable features: the hammerhead ribozyme is a well-studied catalytic RNA that has been kinetically characterized [24], and it has been shown to be efficiently inhibited by aminoglycoside antibiotics [9]; competition with magnesium ions has been suggested to be relevant to aminoglycoside binding [25]; and, importantly, the crystal structures of two hammerhead ribozymes have been reported [26–28]. As we illustrate in this article, by utilizing synthetic chemistry, biochemical assays, molecular modeling and molecular-dynamics simulations, important elements pertinent to the hammerhead–aminoglycoside binding can now be deciphered. A novel recognition model that sheds light on antibiotic-RNA binding is discussed and its potential generality and use in future drug design is evaluated.

Aminoglycoside antibiotics inhibit the hammerhead ribozyme

Figure 1 shows the structure of two representative families of aminoglycoside antibiotics. All members contain the highlighted 2-deoxystreptamine (2-DOS) core. Glycosylation of the 4- and 6-hydroxyl groups with various aminosugars yields the kanamycin family, whereas glycosylation of the 4- and 5-hydroxyl groups of 2-DOS characterizes the neomycin family. The presence of a number of amino groups with a range of pK_a values (5.7–8.8) makes these natural products highly charged at physiological pH [29–31]. The polycationic pseudo-oligosaccharide antibiotics are best known for their ability to interfere with ribosomal function in prokaryotes through binding to the

Figure 1



Representative examples of aminoglycoside antibiotics. The 2-deoxystreptamine (2-DOS) is red and the amino groups are highlighted in blue. Man, mannose.

decoding region on ribosomal RNA [6,32]; this disrupts bacterial protein biosynthesis and confers strong bactericidal properties onto the antibiotics. Recent studies have demonstrated that aminoglycoside antibiotics also bind to the HIV-1 Rev response element (RRE) [11] and the *trans* activating response element (TAR) [12], as well as to numerous ribozymes [7–10].

Stage *et al.* [9] have investigated aminoglycoside antibiotics as inhibitors of the hammerhead ribozymes (Figure 2), and found neomycin B to be a potent inhibitor of the cleavage reaction with a K_I of 13.5 μM. The data indicated that the antibiotics interacted preferentially with the enzyme–substrate complex. This interaction has been suggested to reduce the cleavage rate by stabilizing the ground state of the complex and destabilizing the transition state leading to the phosphodiester cleavage. Comparing the inhibitory activity of various aminoglycosides implied that the number of ammonium groups on the antibiotics is important for RNA binding. By studying the inhibition as a function of pH and Mg^{2+} concentration, it was concluded that the positively charged neomycin inhibits the hammerhead ribozyme by displacing critical Mg^{2+} ions under physiologically relevant conditions [25].

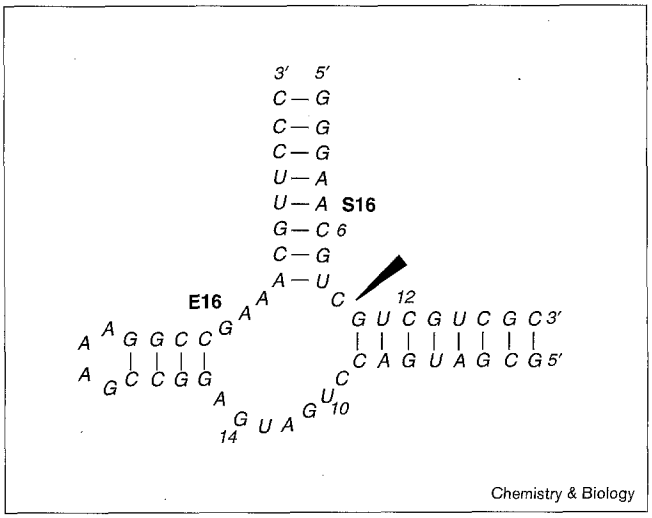
Binding affinities of modified aminoglycosides correlate with the number of positive charges

Further support for the role of electrostatic interactions in RNA–aminoglycoside binding has been obtained by investigating synthetic 'amino-aminoglycosides' [33]. We have replaced the primary CH_2OH group in natural aminoglycosides with a highly basic CH_2NH_2 group (Figure 3). These 'amino-aminoglycosides' have enhanced binding affinity for the hammerhead ribozyme when compared to the parent natural products. Kanamycin A, a poor RNA-binder that contains four amino groups, is converted into a reasonably strong inhibitor upon the incorporation of an additional amino group. Thus, 6″-amino-6″-deoxykanamycin A is as effective as kanamycin B, a natural product containing five amino groups. Similarly, modifying a stronger binder such as tobramycin further enhances its affinity for the hammerhead ribozyme: 6″-amino-6″-deoxytobramycin is approximately fivefold more effective than the parent tobramycin as a ribozyme inhibitor. Even the binding affinity of neomycin B, one of the strongest RNA binders, can be further enhanced by converting it to 5″-amino-5″-deoxyneomycin B. These results suggest that increasing the overall charge of a ligand is an important mechanism for increasing RNA affinity.

Binding affinities correlate with the basicity of the ammonium groups

Tobramycin inhibits the hammerhead ribozyme more effectively than kanamycin B although both compounds contain the same number of amino groups (five), and this trend is seen in other RNA systems that have been explored [7,8,11]. The only difference between the two antibiotics is an additional hydroxyl group at the 3′ position in kanamycin B. How can the presence of a hydroxyl

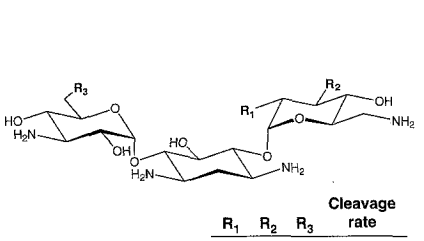
Figure 2



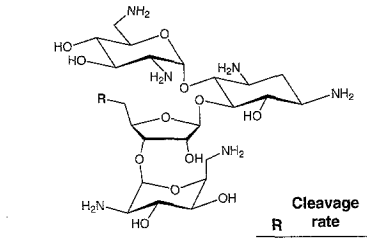
Secondary structure of the hammerhead ribozyme 16 (HH16) showing the 'enzyme' E16 and the 'substrate' S16. The arrowhead indicates the cleavage site.

Figure 3

Parent aminoglycosides and their synthetic 'amino-aminoglycosides' derivatives. The relative ribozyme cleavage rate in the presence of the antibiotics (100 μM for kanamycins, 10 μM for tobramycins and neomycins) is normalized to the ribozyme cleavage rate in the absence of antibiotics [33].



	R_1	R_2	R_3	Cleavage rate
Kanamycin A	OH	OH	OH	**0.77**
6′-Amino-6′-deoxykanamycin A	OH	OH	NH_2	**0.24**
Tobramycin	NH_2	H	OH	**0.68**
6′-Amino-6′-deoxytobramycin	NH_2	H	NH_2	**0.15**



	R	Cleavage rate
Neomycin B	OH	**0.24**
5′-Amino-5′-deoxyneomycin B	NH_2	**0.04**

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group impede RNA binding? We studied a series of deoxygenated tobramycin derivatives in which the hydroxyl groups had been systematically removed while all other functional groups were kept intact [34]. The most potent ribozyme inhibitors are the deoxygenated derivatives lacking the 2″-, 4″- and 4′-hydroxyl groups (Figure 4). In contrast, removal of the primary 6″-hydroxyl group results in a poorer RNA binder than the parent tobramycin. The results indicate that when a hydroxyl group proximal to an amine group is removed, higher inhibitory activity is observed; these observations were attributed to the increased basicity of an amino group in the absence of a neighboring hydroxyl group, similar to the trend observed with aliphatic amines ($HOCH_2CH_2NH_3^+$, pK_a=9.5; $CH_3CH_2NH_3^+$, pK_a = 10.7) [35]. Thus, the deoxygenated aminoglycoside derivatives might have a higher positive charge density at a given pH than their parent natural product. These observations support the critical role of electrostatic interactions in RNA binding, and suggest that altering the pK_a of amino groups is an important mechanism for modulating the affinity of synthetic ligands for RNA.

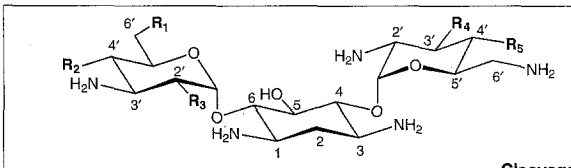
In summary, the studies with synthetically modified aminoglycosides have revealed two consistent trends in aminoglycoside inhibition of the hammerhead ribozyme. Firstly, increasing the number of positive charges on the antibiotics increases their binding affinities. Secondly, a higher basicity of the amino groups (leading to a higher charge density at neutral pH) enhances further the affinities for aminoglycoside binding to the RNA. We will now discuss how these findings can be incorporated into a model developed independently for the recognition of the hammerhead RNA by aminoglycosides.

How do aminoglycosides bind to the hammerhead ribozyme?

Aminoglycosides contain several inter-ring glycosyl bonds about which rotation is allowed, albeit in a sterically hindered fashion. Figure 5a shows the energy map for rotations about the Φ and Ψ torsion angles between rings A and B of neamine, the invariant unit of most active aminoglycoside antibiotics. The map displays two main minima, a broad one (around –150°/–150°) and a sharp one (around +75°/–150°), connected by a shallow valley that marks the path of interconversion between the principal conformers. Thus, variations in those two angles, along with additional steric interactions of further ligands connected to the neamine moiety, lead to numerous conformers of aminoglycosides [36]. The four inter-ammonium distances observed in the accessible solution conformers of aminoglycosides can be parsed into two main sets: two are intra-ring and rather invariant, whereas the other two are inter-ring and rather variable (Figure 5b).

Crystal structure analyses have revealed a number of magnesium ions located within the cavity formed by the deep grooves of stems I and II (which face each other) in the three-dimensional fold of the hammerhead ribozyme [27,28]. The bandwidth of inter-Mg^{2+} distances in solution conformations of the hammerhead RNA was explored

Figure 4



Aminoglycoside	R_1	R_2	R_3	R_4	R_5	Cleavage rate
Kanamycin B	OH	OH	OH	OH	OH	**0.24**
Tobramycin	OH	OH	OH	H	OH	**0.16**
4′-Deoxytobramycin	OH	OH	OH	H	H	**0.053**
6′-Deoxytobramycin	**H**	OH	OH	H	OH	**0.23**
4′-Deoxytobramycin	OH	**H**	OH	H	OH	**0.053**
2′-Deoxytobramycin	OH	OH	**H**	H	OH	**0.027**

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Parent aminoglycosides and their synthetic deoxytobramycin derivatives. The relative ribozyme cleavage rate in the presence of 100 μM antibiotics is normalized to the ribozyme cleavage rate in the absence of antibiotics [34].

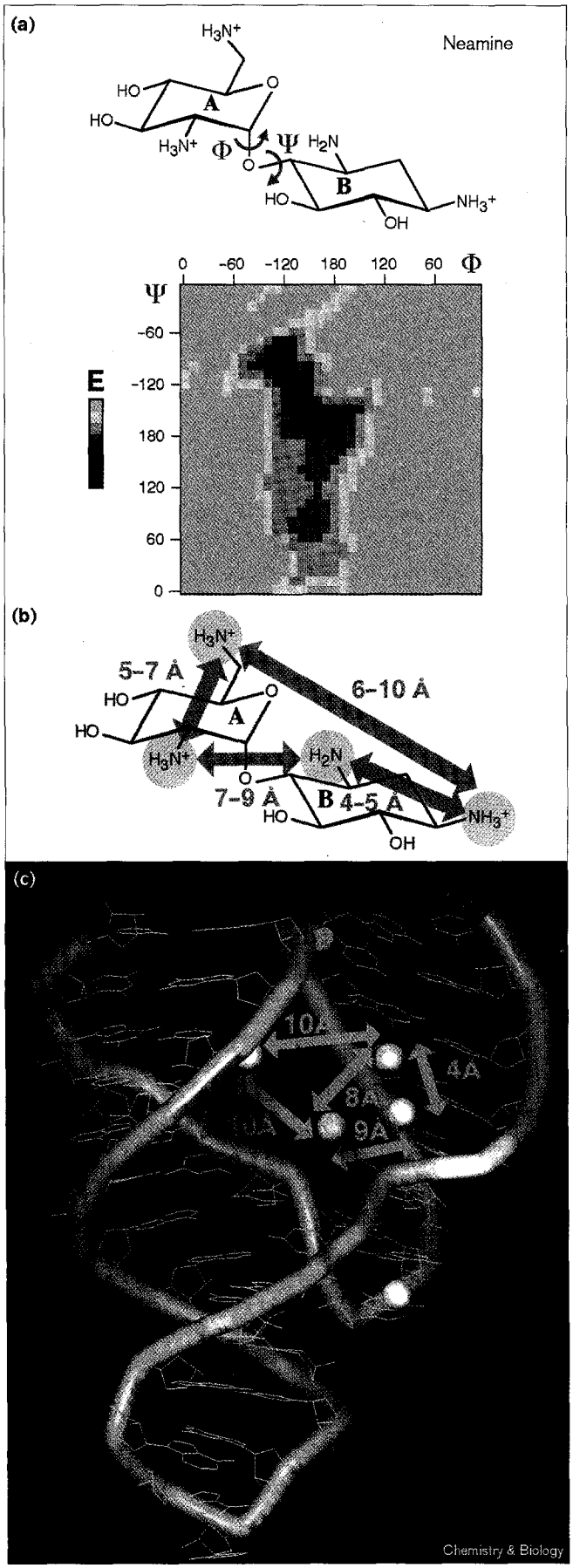


Figure 5

Congruent sets of inter-ionic distances between the solution conformers of aminoglycosides and the hammerhead ribozyme. **(a)** Adiabatic map of the two torsion angles connecting rings A and B of the neamine core common to all aminoglycosides. **(b)** Intramolecular distances between ammonium groups observed in solution conformers of various aminoglycosides [36]. **(c)** Inter-ionic distances between magnesium ions bound in the cavity of the hammerhead ribozyme [28,36,37]. The cleavage site is marked in yellow.

using molecular-dynamics simulations [37]. These calculations revealed a striking congruence between the inter-ionic Mg^{2+}–Mg^{2+} distances in the free ribozyme (Figure 5c) and the intramolecular distances between the positively charged ammonium groups in solvated aminoglycosides (Figure 5b) [36]. It was suggested that the covalently linked array of ammonium groups in the antibiotics is capable of displacing three or four magnesium ions in the hammerhead ribozyme by complementing the negative electrostatic potential created by the RNA fold [36]. Indeed, many solution conformers of different aminoglycosides could be successfully docked with the hammerhead fold in such a way that ammonium groups of aminoglycosides occupy sites of Mg^{2+} ions in the RNA crystal structure (Figure 6) [36]. This hypothesis has been assessed using molecular-dynamics simulations of complexes of the hammerhead RNA and aminoglycoside antibiotics Analysis of the molecular-dynamics trajectories of the drug–RNA complexes revealed that the stable interactions formed between the ammonium groups of the aminoglycosides and the RNA are almost identical (down to an atomic level) to the interactions observed for Mg^{2+} in the hammerhead crystal structure (Figure 6) [36].

Water-mediated contacts contribute to three-dimensional electrostatic complementarity

Water molecules play an important role in mediating interactions between the RNA and both magnesium ions and aminoglycosides (Figure 6b,c). The ability of water molecules to rearrange at the interface between the bound species and the RNA provides the maximum number of hydrogen-bonding contacts at an optimal distance and orientation. The fact that several solution conformations of aminoglycosides could be docked with the hammerhead RNA (and not just one) shows that the relative arrangement of ammonium groups in the drugs provides a set of spatially oriented positive charges that could complement in various ways the field created by the electronegative cavity of the hammerhead RNA (Figure 7). The high-affinity binding of different solution conformations of aminoglycosides is facilitated by the plasticity of the water-mediated contacts between the drug and the RNA. This model suggests a three-dimensional electrostatic complementarity (Figure 7), rather than highly specific contacts between the aminoglycoside and an RNA-receptor site and is in agreement with recent studies [18,33].

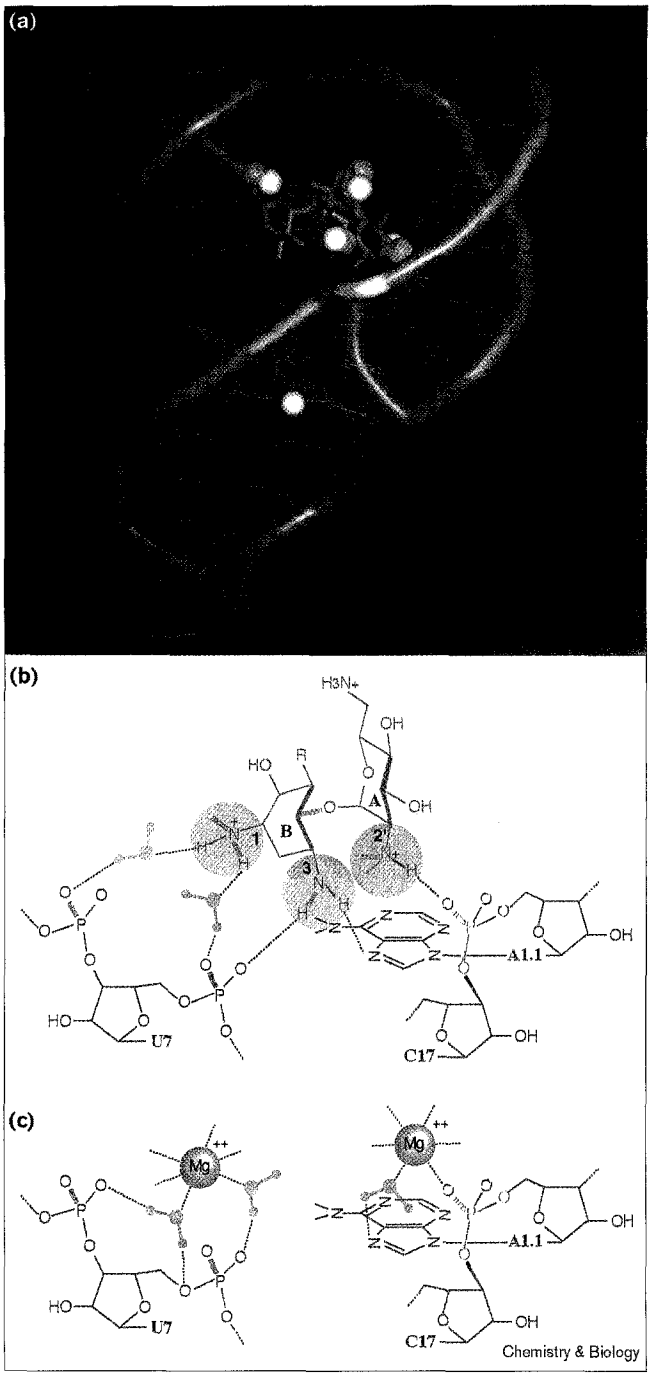
Impact of the recognition model on drug design

The large body of biochemical and structural data available for the hammerhead ribozyme allows the straightforward assessment of hypotheses focusing on drug–RNA interactions. Measuring the inhibitory effects of drugs on the catalytic activity of the ribozyme provides an elegant way of monitoring their RNA-binding ability. Recent efforts in studying the hammerhead–aminoglycoside system have led to a recognition model based on structural electrostatic complementarity: aminoglycosides provide a three-dimensional framework in which combinations of positively charged ammonium groups are arranged to fit the negatively charged metal-ion-binding pockets created by the electrostatic field around the hammerhead RNA fold. The combined experimental and theoretical studies show that the inhibition potential of aminoglycosides is dependent on the following considerations: the number of charged ammonium groups in the aminoglycoside; the basicity of the amines; and the number of different, but energetically similar, solution conformers that can replace effectively several metal ions in the hammerhead RNA. Thus, synthetic organic chemistry, molecular biology and molecular modeling come together to yield an intriguing recognition model that provides new insights into the design of RNA-targeted drugs.

In what ways is the information gained from these studies relevant for explaining the recognition between small molecules and RNA and, ultimately, for designing new drugs? Firstly, the work shows that theoretical approaches are now sophisticated enough to allow the development of recognition models that may be useful as a basis for rational drug design. Secondly, the work stresses the role of three-dimensional electrostatics in drug–RNA interactions: it is the relative spatial positioning of charges that drives both recognition and binding. Thirdly, although the displacement of water molecules and ions has been recognized for some time as an important element in RNA binding, the model presented here suggests that this is not a purely stochastic and haphazard process. Indeed, ions occupy definite positions in an RNA fold and their simultaneous displacement requires a molecular framework of positive charges with appropriate geometry.

The proposed recognition model invokes straightforward strategies for drug design. To start with, the carbohydrate moieties of aminoglycosides constitute exquisite scaffolds around which positive charges, such as ammonium groups, can be organized. The interaction strength between the cationic groups and the RNA host can be modulated by electronegative neighboring substituents such as hydroxyl groups. Furthermore, the enormous versatility in forming glycosidic linkages between the carbohydrate moieties provides an adequate three-dimensional diversity for specifically targeting the various RNA pockets and folds encountered in potential biological targets.

Figure 6



Molecular models of complexes obtained by docking solution conformations of aminoglycosides with the crystal structure of the hammerhead RNA (adapted form [36]). **(a)** Representative complex between neomycin B and the hammerhead. White spheres depict the position of magnesium ions in the crystal structure and green spheres show the ammonium groups in the aminoglycoside. The cleavage site is marked in yellow. **(b)** Interactions between aminoglycosides and the RNA in molecular-dynamics simulations of the docked complexes. **(c)** Interactions between magnesium ions and the RNA in molecular-dynamics simulations of the fully hydrated and neutralized hammerhead ribozyme. In (b) and (c), notice how water molecules (cyan) participate in the binding of the positively charged ions to the RNA.

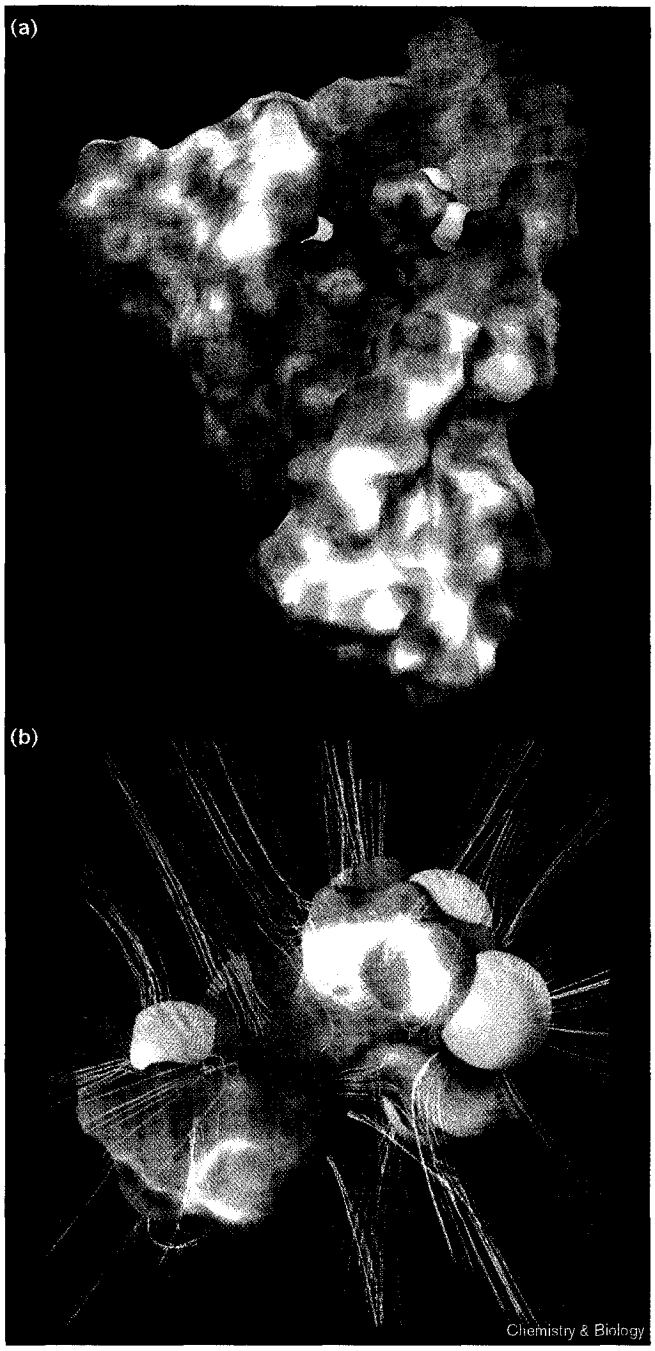


Figure 7

Electrostatic complementarity between the electronegative cavity in the hammerhead RNA and the positively charged aminoglycosides. **(a)** Projection of the charge density on the molecular surfaces of a hammerhead–neomycin complex. Negatively charged surface patches are red, positively charged patches are blue. The positions of magnesium ions, as observed in the crystal structure of the hammerhead RNA, are indicated by light blue spheres. In the modelled complex, metal ions in the cavity are displaced by the aminoglycoside. **(b)** Field lines (yellow) illustrate the gradient of the electrostatic field [38] created by one aminoglycoside conformer. The positions of the displaced magnesium ions are shown as light blue spheres. The conformer and the orientation are the same as in (a) where the aminoglycoside is shown docked with the hammerhead RNA. Note the bundles of field lines emerging from positive surface patches of the aminoglycoside; they point in the directions of the positively charged metal ions that are located in the negatively charged binding pockets created by the RNA fold. The aminoglycoside adopts an orientation inside the cavity of the hammerhead so as to direct its positive charges into the metal-ion-binding sites of the RNA. This figure was made using the program GRASP [39].

Because of their importance for delineating the electrostatic environment of an RNA fold, electrostatic calculations and molecular simulations should be pursued and further developed [38]. Characterization of both the three-dimensional structure and the electrostatic environment of an RNA fold is a prerequisite for choosing the proper molecular architecture and the positioning of charged groups in the design of a drug specific for a given RNA target. The promiscuous binding of aminoglycoside antibiotics to the hammerhead RNA arises from their conformational flexibility, which allows them to 'remodel' according to the RNA-binding surface. Although the ensuing entropic losses are compensated for by the simultaneous displacement of several ions and the rearrangement of water molecules at the interface, interactions with multiple partners is not desirable in drug design. Promising new directions include the design and synthesis of more rigid aminoglycosides and novel hybrid molecules that combine several different binding modes, such as ionic interactions and intercalation. Due to the complexity of the problem and the challenges it presents, we will have to rely on the ingenuity of chemists and molecular modelers for the design and synthesis of the next generation of specific RNA binders.

Acknowledgements

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