Interactions of aminoglycoside antibiotics with rRNA

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Abstract

Aminoglycoside antibiotics are protein synthesis inhibitors applied to treat infections caused mainly by aerobic Gram-negative bacteria. Due to their adverse side effects they are last resort antibiotics typically used to combat pathogens resistant to other drugs. Aminoglycosides target ribosomes. We describe the interactions of aminoglycoside antibiotics containing a 2-deoxystreptamine (2-DOS) ring with 16S rRNA. We review the computational studies, with a focus on molecular dynamics (MD) simulations performed on RNA models mimicking the 2-DOS aminoglycoside binding site in the small ribosomal subunit. We also briefly discuss thermodynamics of interactions of these aminoglycosides with their 16S RNA target.

Introduction

Aminoglycosides are antibiotics targeting ribosomes that interfere with bacterial protein synthesis. They are often last chance antibiotics used to cure hospital-acquired infections caused mainly by aerobic Gram-negative bacteria [1]. Aminoglycosides are still valuable drugs used to treat such infections as sepsis, tuberculosis, tularemia, brucellosis and nosocomial respiratory tract infections. They also provide synergistic bactericidal activity in combination with antimicrobials that inhibit bacterial cell wall synthesis [2]. Moreover, they show potential in the treatment of HIV-1 infected patients and human genetic disorders [3]. Aminoglycosides are injected because they are poorly absorbed from the gut. Oral administration can be used in hepatic encephalopathy. However, prolonged use of high aminoglycoside doses may result in their ototoxicity and nephrotoxicity.

The main resistance mechanism is related to deactivation of aminoglycosides through their chemical modification by bacterial aminoglycoside modifying enzymes [4]. Resistant bacterial strains have plasmids containing genes encoding these enzymes. However, enzymatic modifications by ribosomal methyltransferases or mutations of the target (even though rare) also contribute to resistance. In addition, bacteria may decrease aminoglycoside uptake by reducing membrane permeability or actively pump them out of the cell.

2-Deoxystreptamine (2-DOS) aminoglycoside antibiotics bind to decoding A-site in helix 44 of 16S RNA in proximity to mRNA codon and anti-codon of the aminoacyl-tRNA. They interact with and hinder the dynamics of two functional A-site adenines (A1492 and A1493, *Escherichia coli* numbering) that act as a switch ensuring the accuracy of decoding (Figure 1). Binding of 2-DOS aminoglycosides locks these adenines in a flipped-out state which promotes decoding errors by allowing incorporation of near-cognate and non-cognate tRNAs [3]. However, 2-DOS aminoglycosides also interact with Helix 69 of 23S rRNA in the large subunit. These two binding sites are conserved elements of ribosomes and form a flexible intersubunit bridge responsible for collective functional motions of the subunits. Some 2-DOS aminoglycosides were shown to alter the dynamics of this bridge and to further affect translocation of tRNAs and subunit rotation [5]. Also, aminoglycosides interfere with the formation of ribosome subunits by stabilizing subunit precursor particles [6].

From the chemical point of view, 2-DOS aminoglycosides are pseudo-oligosaccharides composed of two to five rings connected by flexible glycosidic linkers (Figure 2). Depending on the position of these linkers, aminoglycosides are divided into 4,5- or 4,6-disubstituted 2-DOS. Most pK_as of their amino groups are above 7 making them positively charged at physiologic pH [7]. Due to such polycationic nature, aminoglycosides have high affinity for the negatively charged phosphates in the nucleic acid backbone. Thus they bind to a variety of RNA targets [7,8], including human ribosomes, especially the mitochondrial ones whose small subunit A-site has a similar secondary and tertiary structure to the bacterial one [9] (Figure 1). This was confirmed by measuring the activity of 2-DOS aminoglycosides against bacterial cells but with ribosomes containing different A-site sequences [10]. Therefore, aminoglycosides are not sufficiently selective, which results in their adverse side effects [11]. Additionally, one can be predisposed to aminoglycoside-induced deafness, which is associated with the A1555G mutation in the mitochondrial 12S rRNA [12] (Figure 1).

2-DOS aminoglycoside binding site in the ribosome is composed only of RNA as confirmed by the crystal structure of the 30S subunit in the complex with

Key words: aminoglycoside antibiotics, electrostatic interactions, molecular docking, MD simulations, rRNA, thermodynamics.

Abbreviations: 2-DOS, 2-deoxystreptamine; ITC, isothermal titration calorimetry; L-HABA, L-(−)-γ-amino-α-hydroxybutyric acid; MD, molecular dynamics.

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Figure 1 | **The position of 2-DOS aminoglycoside binding site in the 30S subunit (in pink, PDB code: 1IBK [14])**
Lower inset shows the most probable sequences of this site in human and bacterial ribosomes. Paromomycin is shown as green van der Waals spheres, mRNA fragment is in red.

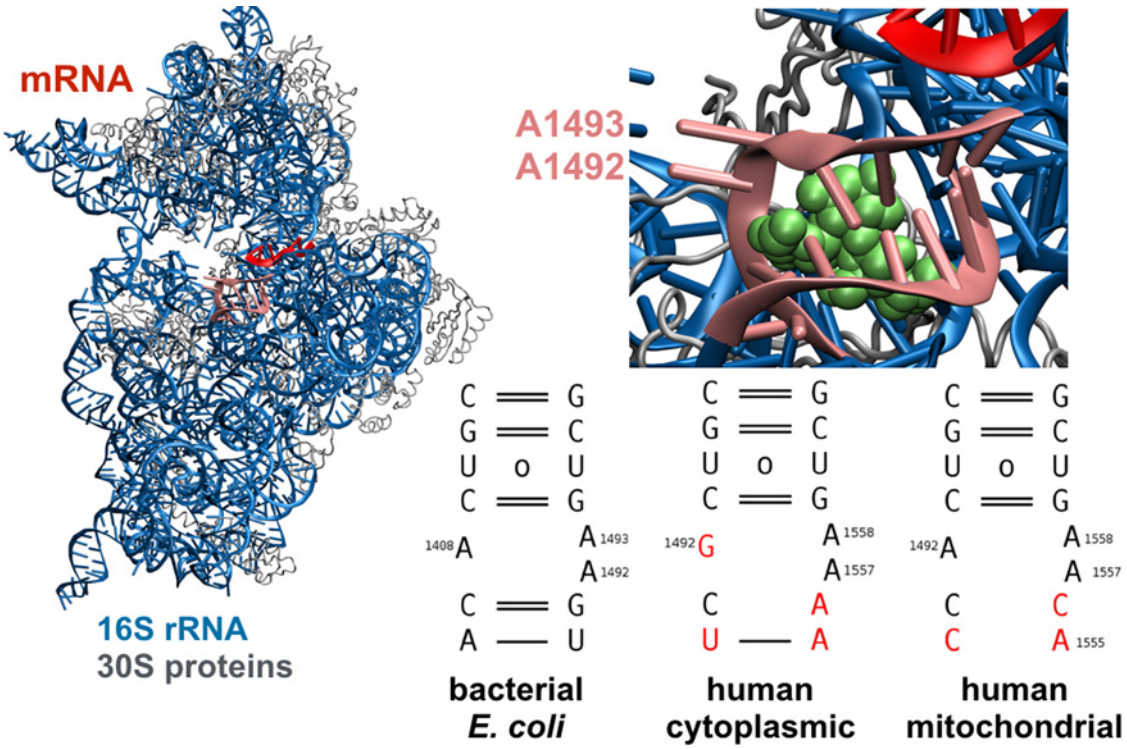


Figure 2 | Examples of 4,5 and 4,6-disubstituted 2-deoxystreptamines

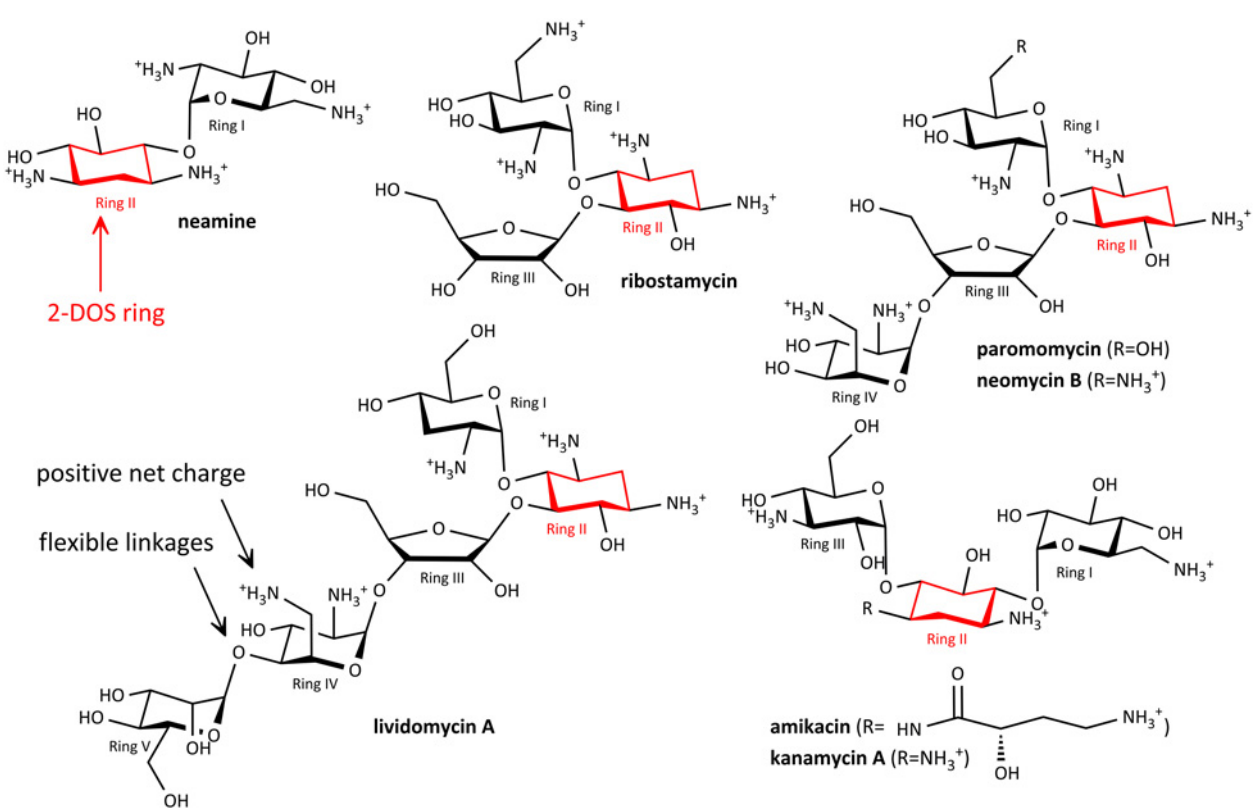
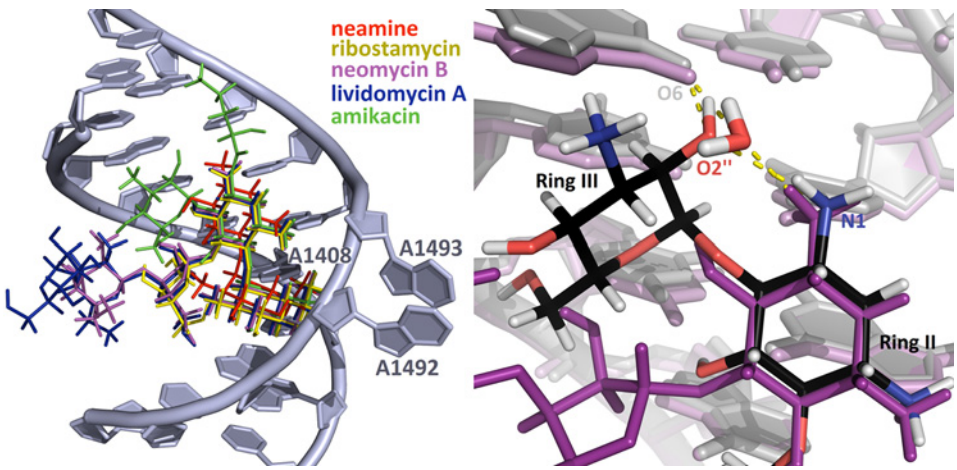


Figure 3 | 2-DOS aminoglycoside binding mode in the model of the decoding A-site

Left: Selected 2-DOS aminoglycosides superimposed on the RNA model of the 16S RNA A-site. PDB codes of the crystal structures of the complexes: neamine (2ET8 [37]), ribostamycin (2ET5), RNA (in grey) and neomycin B (2ET4), lividomycin A (2ESJ), amikacin (2G5Q [68]). Only one A-site of the crystallized duplex is shown. Right: Superimposed complexes with neomycin B (purple) and kanamycin A (coloured by atom name) with RNA shown in pink and grey respectively. The water molecule present in the neomycin B complex mediates hydrogen bonds (yellow dashed lines) between the N1 amino group of aminoglycoside ring II and O6 atom of the base G1405. Similar hydrogen bonds are made by the O2″ hydroxy group from kanamycin's ring III.



paromomycin [13,14]. Also, RNA oligonucleotide models with sequences corresponding to 16S RNA decoding A-site were crystallized and shown to mimic well this rRNA bulge and aminoglycoside mode of binding [15] (Figure 3). The structural data confirmed that such aminoglycoside binding site forms also without the ribosome context. This 22 base pair RNA duplex contains two symmetric aminoglycoside binding sites (bulges) and is often used as a starting point for molecular dynamics (MD) simulations [16]. Aminoglycosides anchor to their rRNA target through a neamine core (rings I and II in Figure 2). Electrostatics is believed to play a leading role in aminoglycoside short-range interactions with nucleic acid targets [17–19], as well as in aminoglycoside diffusion towards the A-site [20,21]. However, also the role of the aminoglycoside-aromatic CH/π stacking contribution should not be underestimated [22].

Understanding the interactions between 2-DOS aminoglycosides and their RNA target at atomic detail is important to design antibiotic modifications or new scaffolds that would increase their selectivity towards the bacterial target. The ribosome is flexible, both locally (decoding site adenines are in equilibrium between extra and intra-helical states) and globally (the ribosome undergoes concerted motions during entire translation process, especially in the polypeptide elongation phase). Simulations can complement experiments to give insight into the dynamics at atomic resolution. We review computational approaches, mainly MD simulations, which used models of aminoglycoside binding site in 16S RNA. MD is a common method to study internal flexibility of molecules at atomic resolution and surrounded by explicit water molecules and ions. Newton's equations of motions are numerically solved in femtosecond time steps to obtain a trajectory of motions, i.e. the positions and velocities of atoms as a function of time. The classical mechanics based potential energy of the molecule is a sum of simple analytic functions with semi-empirical parameters [23]. MD approach, even though conceptually simple, has proven useful to determine conformational states of biomolecules around starting structures.

Thermodynamics of 2-DOS aminoglycoside interactions

A single-stranded 27-mer RNA hairpin containing one bulge has been often used in solution studies as a mimic of the small subunit decoding A-site binding 2-DOS aminoglycosides [16,24]. The thermodynamic binding data for this RNA oligonucleotide showed that the equilibrium dissociation constants for 2-DOS aminoglycosides are in the micromolar range and correlate with the total charge of the antibiotic [19,25–28]. Moreover, aminoglycoside binding at pH above 5.5 is linked with proton uptake by their amino groups [29]. Overall, in accord with the positive net charge of these antibiotics, increasing ionic strength or pH decreases their binding affinities to RNA. Also, in our study on the electrostatics of aminoglycoside – 16S RNA A-site crystallographic complexes, we observed correlations

between the experimentally determined binding free energies and electrostatic contribution to binding [30]. However, in isothermal titration calorimetry (ITC) studies, depending on buffer solutions and pH conditions, for some 2-DOS aminoglycosides, a favourable entropic contribution (positive $T\Delta S$) to binding to the decoding A-site model was observed [9,27,28,31,32]. Since ITC gives information on the generation or release of heat occurring in the entire process in the sample cell, it is difficult to assess what contributes to the observed positive entropy term. It could come from conformational changes of aminoglycoside, RNA or release of ions upon binding. We have also observed a positive $T\Delta S$ in the ITC studies of amikacin, a semi-synthetic derivative of kanamycin A, which possesses a unique L-HABA (L-(−)-γ-amino-α-hydroxybutyric acid) extension [32] (Figures 2 and 3). Amikacin, probably due to this L-HABA tail, is quite resistant to enzymatic modifications by bacterial enzymes. However, it seems that this tail has also another role that of balancing the enthalpy–entropy contributions to binding. With MD simulations we found that amikacin's tail is more flexible in the complex with RNA than in water solution [32]. The hydrogen bonds formed by this tail observed in the crystal structure turned out to be weak and transient in MD simulations. Since the presence of amikacin did not affect the UV-melting profiles and circular dichroism spectra of the oligonucleotide A-site model, it could be that the positive $T\Delta S$ arises also from the L-HABA tail.

Hydration patterns in aminoglycoside binding site

In the crystal structures of 16S RNA A-site models in the complexes with 2-DOS aminoglycosides many water-mediated hydrogen bonds were found and simulations have proven their importance in the interactions with RNA [33–35]. For example, a water molecule bridges the interactions of the antibiotic with a uracil pair U1406/U1495 (Figure 1) and contributes to the stability of the complexes [34,36]. An MD study of the A-site oligonucleotide model in the complex with paromomycin showed that the neamine core forms crucial hydrogen bonds stabilizing this antibiotic, whereas two other rings interact more weakly and transiently with RNA [33]. Also, many water-mediated contacts between paromomycin and RNA were found stable in these simulations.

The analysis of crystallographic structures of the rRNA complexes with twelve 2-DOS aminoglycosides showed repeated water-mediated hydrogen bond patterns [30]. Therefore, the water molecules that interact strongly with aminoglycosides and weakly with RNA could be mimicked with modified antibiotic groups. A pattern showing the idea of using the hydrogen bonds of mediating waters for designing new modifications of aminoglycoside functional groups is presented in the right panel of Figure 3. In the crystallographic structures of the aminoglycoside binding site RNA models [37] a hydroxy group of kanamycin A mimics the water molecule present in the complex with neomycin B.

Flexibility of aminoglycoside binding site models from MD simulations

Even though all-atom MD simulations of the whole ribosome or its subunits have been performed to study mRNA decoding and tRNA translocation, e.g. [38,39], MD studies of the RNA models of aminoglycoside binding site allowed focusing on local dynamics and adenine flipping on longer timescales. Classical MD studies of 16S RNA A-site models confirmed the flipping motion of A1492/93 [34,40,41]. MD simulations with enhanced sampling approach characterized the thermodynamics of adenine mobility and estimated the energetic barrier for adenine flipping in the range of 0.5–5 kcal/mol [42]. These two bases were found to flip in and out of the bulge on a timescale faster than aminoglycoside binding suggesting a stochastic gating mechanism where the ligand does not induce flipping out of A1492/93 but only traps them in a flipped out state [43]. Another targeted MD study in combination with approximate free-energy calculations (with a molecular mechanics/Poisson–Boltzmann method) showed that A1492/93 preferred the flipped-out state [41].

The dynamics of the small subunit A-site RNA models with single and double-point nucleotide substitutions were studied to understand the effect of mutations on aminoglycoside short-range interactions with RNA [34,44]. These studies showed that nucleotide substitutions may affect local dynamics of A1492/93 and paromomycin binding mode, as well as hydration patterns and ionic distributions around RNA bulge. Specifically, MD simulations of the A1408G A-site variant that is characteristic of eukaryotic cytoplasmic ribosomes (Figure 1) showed that (i) more Na^+ ions gather in the binding site and (ii) A1492/93 interact more strongly with G1408, which may explain why bacteria with A1408G substitution are resistant to aminoglycosides [45].

MD simulations were also applied to investigate the reasons for different selectivities of 2-DOS aminoglycosides towards decoding A-sites of various organisms [36]. In the human variant we observed flipped-out conformations of A1491 resulting in narrowing of the major groove and frequent exchange of conformations within the U1406/U1495 (Figure 1). These factors may explain the slightly lower (one-fold) binding affinity of 2-DOS aminoglycosides towards the human cytoplasmic A-site variant [9].

Even though the 2-DOS aminoglycoside binding site is bare of proteins, amino acid alterations in a nearby ribosomal protein S12 affect activity of these antibiotics. S12, together with the S4 and S5 ribosomal proteins, contributes to the fidelity of decoding. Certain mutations in the S12 protein (such as K42A and R53A) inactivate paromomycin even if it is already bound to the ribosome [46]. These S12 mutants also make ribosomes hyper-accurate. With MD simulations of a ribosome fragment, we found that these S12 mutations affect the mobility of A1492/93 by allowing the interactions

of A1492 with a flexible K43 [47]. Therefore, the miscoding effect of paromomycin maybe cancelled because A1492/93 acquire conformations less favourable for aminoacyl-tRNA accommodation.

Other computational methods in the studies of 2-DOS aminoglycosides and their 16S RNA target

Apart from classical all-atom MD simulations, other techniques have been also applied to study the interactions of 2-DOS aminoglycosides and their 16S RNA target. One study used an empirical potential to calculate the contributions of various 2-DOS aminoglycoside rings to the interactions with rRNA [48]. For example, for paromomycin and neomycin B large contributions of rings I, II and IV were found, with a much smaller contribution of ring III (Figures 2 and 3). The interaction energies were also calculated with *ab initio* quantum mechanical calculations [49].

The Poisson–Boltzmann model of electrostatics was used to verify the importance of electrostatic contribution to binding [18,19]. The study used the whole 30S subunit but the calculations did not explicitly include its dynamics. Reduced representations of the antibiotics were used to detect how they diffuse towards the RNA target. These further confirmed the importance of electrostatics not only in the local short-range interactions but also in the first steps of the binding process, i.e. pathways of association towards the target [20,21].

Atomistic level description of aminoglycoside–RNA interactions is important to aid in the design of new compounds. Predicting the most optimal binding mode of the complex is termed docking. Docking involves searching for and scoring conformations of the complex, typically based on the molecular mechanics potential energy function. Docking is necessary after virtual screening of chemical databases to predict and rank the best binding poses of modified or new compounds. Approaches to predict the 2-DOS aminoglycoside poses in the 16S RNA decoding A-site mimics were tried. However, modifications of the docking algorithms that included the assessment of RNA flexibility or implicit treatment of bridging waters were necessary [50–52]. Since aminoglycosides are charged and flexible, some interactions are water mediated, and ions are expelled upon binding, standard docking protocols do not work for these molecules. Therefore, aminoglycosides are often included in the test sets for evaluation of the scoring functions in docking [51,53–55].

Restricting aminoglycoside flexibility to avoid resistance and toxicity

Finding new modifications of natural or semi-synthetic aminoglycosides could help overcome bacterial resistance and toxicity problems. It would be desirable to modify aminoglycosides in such a way to increase their specificity towards pathogenic bacterial ribosomes and to minimize their affinity towards human, specifically mitochondrial, ribosomes. Also, modified aminoglycosides should either inhibit or reduce the activity of aminoglycoside modifying enzymes. Numerous such efforts have been undertaken, e.g. [3,56–59] but without spectacular clinical advancements. However, to increase aminoglycoside selectivity towards bacterial ribosomes, it is essential to understand the dynamics of aminoglycosides and their binding site since both are inherently flexible in solution. Formation of complexes, with some exceptions, e.g. of the L-HABA tail or ring IV in paromomycin, reduces aminoglycoside mobility. To narrow the RNA target selection, the conformational freedom of 2-DOS aminoglycosides around glycosidic bonds has been restricted. Conformationally constrained neomycin B and paromomycin analogues have been developed. Even though the affinity of these analogues towards the A-site model decreased, it did not affect binding to a HIV-1 RNA fragment [60]. So this study has shown that this direction could be pursued further to reduce off-target effects.

The ribosome A-sites are conserved so it is difficult to propose selective aminoglycoside modifications but a possible route could be to either design sequence-specific aminoglycoside extensions [61,62] or to combine aminoglycosides perhaps with ribosome inhibitors that target nearby sites. For example, neamine and neomycin B dimers have been already explored to restrict the freedom of linkers, avoid enzymatic modifications and decrease the doses. Neamine dimers were shown to have comparable activity to that of single neamine in *in vitro* translation and antimicrobial assays against *E. coli* [63] but most importantly inhibited the activity of aminoglycoside modifying enzymes. Neomycin B dimers bound with higher affinity to A-site oligonucleotide models and depending on the linker preferred bacterial over the human model [64]. However, their antibacterial activity was weaker than of single neomycin. Also, the neighbouring binding cleft of hygromycin B (which binds at the site of the upper stem in the secondary structure models shown in Figure 1) was used to extend paromomycin to occupy a larger moiety [65]. Recently, aminoglycosides were conjugated to silica nanoparticles and have shown antimicrobial activity against clinical resistant strains and also low cytotoxicity [66]. Regrettably, the quest for new aminoglycoside analogues with enhanced antibacterial properties still resembles a random walk and complex low-yield chemical synthesis of semi-synthetic analogues precludes high-throughput studies on many compounds.

Conclusion

Computational modelling of the dynamics of 16S rRNA fragments mimicking the 2-DOS aminoglycoside binding sites helps understand their interactions with RNA and explain experimental data. Specifically, all-atom MD simulations in explicit solvent capture the flexibility of A1492/93, the importance of the neamine core, give insight into the hydration and ionic patterns, as well as give implications

how nucleotide substitutions change these interactions. The advances in both computer power and potential energy functions for nucleic acids [67] should make MD simulations more routine to use them in flexible docking studies. Apart from still insufficient sampling, which now only reaches the experimental timescales for the model systems of the size described here, there is still a need to improve the models for divalent ions since they both contribute to the dynamics of RNA and charged aminoglycoside binding.

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