Crystal Structure of Geneticin Bound to a Bacterial 16 S Ribosomal RNA A Site Oligonucleotide

Quentin Vicens and Eric Westhof* 

Institut de Biologie Moléculaire et Cellulaire du CNRS 
Modélisation et simulations des Acides Nucleïques 
UPR 9002, Université Louis Pasteur, 15 rue René Descartes 
67084 Strasbourg Cedex 
France

Aminoglycosides are antibacterial molecules that decrease translation accuracy by binding to the decoding aminoacyl-tRNA site (A site) on 16 S ribosomal RNA. We have solved the crystal structure of an RNA fragment containing the A site bound to geneticin at 2.40 Å resolution. Geneticin, also known as G418, is a gentamicin-related aminoglycoside: it contains three rings that are functionalized by hydroxyl, ammonium and methyl groups. The detailed comparison of the distinctive behaviour of geneticin (binding to pro- and eukaryotic A sites) with the crystallographic, biochemical and microbiological results obtained so far for aminoglycoside-A site complexes offers new insights on the system. The two sugar rings constituting the neamine part common to most of the aminoglycosides bind to the A site, as already observed in the crystal structures solved previously with paromomycin and tobramycin. The essential hydrogen bonds involving ring I (to A1408) and ring II (to the phosphate oxygen atoms of the bulged adenine bases 1492 and 1493 and to G1494) are conserved and additional contacts are observed from ring III (to phosphate oxygen atoms of G1405 and U1406). The present work illustrates a molecular basis of the range in sensitiveness exhibited by geneticin towards common point A site mutations associated to resistance phenotypes. In addition, analysis and comparisons of the structures cast light on the role played by the conserved U1406·U1495 pair in the recognition of the A site by aminoglycosides.

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Introduction

In recent years, an escalating number of three-dimensional NMR and X-ray structures of RNA–ligand complexes displaying atomic details of natural and artificial RNA targets bound to antibiotics (macrolides,1,2 tetracyclines,3,4 aminoglycosides,5–9 and newly designed molecules10 has been published. The goal of these efforts is to understand the physico-chemical rules controlling the specific binding modes of these inhibitors to RNA with the hope of designing new compounds that could help circumvent the antibiotic resistance crisis.

Aminoglycosides are positively charged sugar derivatives that possess several ammonium and hydroxyl functionalities.11,12 On the basis of their chemical structures, the aminoglycosides can be split into two main sub-classes: one that incorporates 2-deoxystreptamine (2-DOS) derivatives and one that does not. The first group can be subdivided into three sub-classes depending on the substitution pattern of the 2-DOS ring. The 2-DOS ring can be functionalized at different positions: at position 4 (e.g. for apramycin), at positions 4 and 5 (e.g. for paromomycin and neomycin) or at positions 4 and 6 (e.g. for tobramycin and genetricin) by sugar rings bearing different ammonium and methyl groups.13 In addition to the 2-DOS ring (ring I), the 4,5- and 4,6-disubstituted aminoglycosides have ring I in common. These two rings constitute the neamine moiety (Figure 1).

Aminoglycosides interfere with protein synthesis by binding to different regions of the 30 S ribosomal particle depending on their chemical structure.7 The 4-substituted, the 4,5- and the 4,6-disubstituted aminoglycosides are micromolar ligands of the eubacterial ribosomal decoding aminoacyl-tRNA site (A site).15–17 Among them, the 4,6-disubstituted geneticin, also known as...
gentamicin G or G418, has been shown to be toxic (like paromomycin in some instances) to eukaryotic organisms such as unicellulars, protozoans, worms, yeasts and mammalian by binding to the 80 S ribosome. Although with some limitations due to its toxicity to human ear and kidney cells, geneticin is used in medicine as an anti-parasitic agent. Some medical efforts are being pursued in the cure of infections by parasites fatal to people suffering from AIDS. Moreover, administration of geneticin or related gentamicins has proven helpful in the treatment of patients suffering from genetic disorders. In molecular biology, geneticin is routinely employed as a selection marker in eukaryotic cells transfected with a gene coding for an enzyme that inactivates the aminoglycoside. These various applications illustrate that geneticin retains a high biological and pharmaceutical interest despite the wide-spread aminoglycoside resistance and toxicity phenomenon.

In the 1970s, the differences in the aminoglycoside activity on protein synthesis were shown to be related to the number and location of the functional groups on the neamine moiety. The first molecular insights into the aminoglycoside binding mode were obtained in the late 1990s by nuclear magnetic resonance (NMR) of paromomycin and gentamicin C1a bound to a 27 nucleotide stem-loop containing the A site. In the last two years, X-ray crystal structures were solved for paromomycin bound to the 30 S ribosomal particle, in the absence and in the presence of cognate tRNA and mRNA. We have obtained, at 2.50 Å and 2.54 Å resolution, respectively, the crystal structures of paromomycin and tobramycin bound to 40 nucleotide RNA constructs containing the A site. The fact that the aminoglycosides bind similarly to the deep groove of the A site in the crystal structures of the complexes with the 30 S particle and with the small RNA fragment demonstrates that the results of our crystallographic studies are biologically relevant. Besides, the neamine moiety interacts with the conserved nucleotides of the A site in the same way for the 4,5- and 4,6-disubstituted compounds.

Here we report the structure of geneticin (Figure 1) bound to the eubacterial ribosomal
decoding A site at 2.40 Å resolution. Whereas the common neamine moiety binds similarly to the A site in the paromomycin, tobramycin and geneticin complexes (referred to as P/A, T/A and G/A complexes, respectively, in the following), contacts specific to geneticin are observed for ring III. The comparisons of the precise interactions involving the aminoglycoside functional groups and the A site in the several crystal structures of the different complexes help to unravel the contribution of each interaction to the binding. Furthermore, the combination of microbiological data, phylogenetic analysis and investigation of the present crystal structure supplies molecular details into the origin of the geneticin-induced toxicity. The rationalization of these results indirectly offers new clues for the design of active compounds.

Results

Overall structure of the G/A complex

RNA fragments incorporating the eubacterial A site proved to be successful in the crystallization of the P/A and T/A complexes.\(^8\)\(^9\) For the G/A complex, narrow plates suitable for X-ray data collection were obtained using the sequence containing a 5'-cytosine overhang\(^8\) (Figure 2(a) and (b)). The space group is \(P_2_1\), (compared to \(P_2_1,2,2\), for the P/A complex and \(P_2_1\), for the T/A complex) and the cell parameters are similar to the parameters of the T/A structure (Tables 1 and 2). This structure was therefore chosen to solve the structure of the G/A complex by molecular replacement at 2.40 Å resolution. The final \(R\) and \(R_{free}\) values are 22.4% and 25.0%, respectively (Table 1).

As in the P/A and T/A structures, the RNA oligonucleotides of the G/A complex form a double helix containing 16 Watson–Crick pairs, two U·U pairs, two unpaired and four bulged adenine bases (Figure 2(c)). The neamine moiety (rings I and II) binds similarly to the two A sites of the RNA fragment (Figures 3 and 4). The superimposition based on the atoms common to tobramycin and geneticin gives a root-mean-square (RMS) deviation of 0.2 Å. The binding of the neamine moiety compels adenine bases 1492 and 1493 of each site to swing away from the helix allowing them to form base-triples with G-C pairs of symmetry-related complexes. These packing contacts are analogous in the three complexes solved. As noticed previously,\(^8\)\(^9\) one of the two sites (site 1) is better defined than the other one (site 2) (Figure 3). However, unexpectedly, the electron density is not as poor in site 2 of the G/A complex as in site 2 of the T/A and P/A complexes, so that alternate conformations (referred to as conformers 1 and 2) could be refined for residues 1492–1494 (Figure 3(b)). As a result, the \(B\)-factors, which were above 100 Å\(^2\) in site 2 at the beginning of the refinement, decreased to

![Figure 2](image-url). Secondary and 3D structures of the geneticin complex. (a) Secondary structure of the A site from the *Escherichia coli* 16S ribosomal RNA. (b) Secondary structure of the crystallized RNA fragment. The two A sites are boxed and the 5'-cytosine overhangs not observed in the electron density are shaded. (c) Crystal structure of the RNA fragment bound to geneticin. The structure is colored according to the \(B\)-factor values, from blue (= 30 Å\(^2\)) to red (= 90 Å\(^2\)) through white (= 60 Å\(^2\)).
The 5' cytosine overhangs are disordered and account for the highest B-factors of the structure observed for the guanine bases of the terminal base-pairs (90–104 Å²).

Detailed interactions between geneticin and the A site

A subset of the hydrogen bonds observed from the functional groups common to the three antibiotics to the A site RNA are identical (Supplementary Material). In site 1 and site 2 (conformer 1), geneticin makes 14 direct hydrogen bonds to base atoms and phosphate oxygen atoms of the A site (Figure 5). Ring I intercalates inside the helix by flipping out adenine bases 1492 and 1493 and by stacking against G1491 (Figure 5(f)). Two hydroxyl groups make hydrogen bonds to the phosphate oxygen atoms of the bulged adenine bases whereas the two other hydroxyl groups contact the Watson–Crick sites of the unpaired adenine 1408 (Figure 5(e)). In comparison to tobramycin and paromomycin, the exocyclic C6 is chiral for geneticin (Figure 1). It is nevertheless oriented as in the P/A complex with O6 contacting N1 of A1408. The additional methyl group C7 points to the middle of the G1491-C1409 pair and induces a higher rise and a smaller propeller-twist angle of that base-pair (212° compared to 223° and −19° for the T/A and P/A complexes) (Figures 4(c), 5(e) and (f)). As in the T/A and P/A complexes, the central ring II makes intramolecular hydrogen bonds to the other rings and ammonium N3 is

Table 1. Crystallographic statistics

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<th>Site 2-containing part (residues 11–22 and 24–33)</th>
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<tr>
<td>R_free (%)</td>
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* Values for last shell are given in parentheses.
** R_sym = Σ[I - 〈I〉]/Σ[I], where I is the measured intensity of each reflection and 〈I〉 is the intensity averaged from multiple observations of symmetry-related reflections.
*** R-factor = 〈|F_o| - |F_c|〉/Σ|F_o|.

60–80 Å², values higher than those in site 1 (30–55 Å²; Figure 2(c)). The 5' cytosine overhangs are disordered and account for the highest B-factors of the structure observed for the guanine bases of the terminal base-pairs (90–104 Å²).

Table 2. Characteristics of the three complexes

<table>
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<th>G/A complex (present work)</th>
<th>T/A complex</th>
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<td>a = 33.0, b = 45.9, c = 95.3</td>
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<td>21.4/26.4</td>
<td>20.6/24.7</td>
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<td>Nature of the 5’ overhang</td>
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<td>UpU</td>
<td>UpU</td>
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<td>2-DOS sub-class</td>
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<td>4,6-Disubstituted</td>
<td>4,5-Disubstituted</td>
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<tr>
<td>Number of positive charges of the antibiotic around neutral pH values</td>
<td>4</td>
<td>5</td>
<td>5</td>
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fully dehydrated. A direct contact from ring II to O4 of U1495 stabilizes the U1406-U1495 pair in a shifted form. In addition, the shifted geometry is maintained by a supplementary contact from O4'' to O2P of U1406 (Figure 5(c)). This additional contact provokes a 0.8 Å shortening of the C1'-C1' distance (from 10.6-10.5 Å in the T/A and P/A complexes to 9.8 Å in the G/A complex) that is likely responsible for the absence of the water molecule observed in the other structures between N3 of U1406 and O2 of U1495 (Figure 4(d)).

The differences in chemical groups between geneticin and the related 4,6-linked tobramycin are essentially located on ring III. The hydroxyl group O2'' and the ammonium group N3'' are conserved and make similar contacts to the Hoogsteen sites of G1405 (Figure 5(b)). In addition, N3'' makes one hydrogen bond to the O2P of the same guanine. Geneticin and tobramycin are epimeric for hydroxyl O4''. Therefore, whereas O4'' is not in a position to make any contact to RNA in the T/A complex, it makes a hydrogen bond to the O2P of U1406 in the G/A complex (Figure 5(c)). Ring III bears also two methyl groups that point away from the RNA. Finally, ring III of geneticin does not contain the hydroxyl O6'' present in tobramycin (interestingly, in the tobramycin complex, O6'' was not involved in any contact).

In site 2, the alternate conformation 2 shows displacements of the phosphate oxygen atoms and of the bases that modify the interactions between rings I and II of geneticin and the phosphate groups of residues 1492–1494 (Figures 3(b) and 6). The major displacement is observed for adenine 1493. The hydrogen bond from O4 of ring I to O2P (A1493) is replaced by a hydrogen bond to a water molecule (Figure 6).

Despite the slightly higher resolution obtained for the G/A complex (2.40 Å) (Table 2), fewer solvent molecules were refined in the final model (40 against 54 and 76 for the P/A and T/A complexes, respectively). Consequently, some of the water-mediated contacts that were observed from ring I

Figure 3. Stereo views of the experimental electron density maps corresponding to the deep grooves of site 1 (a) and site 2 (b). The RNA (yellow), the geneticin (magenta) and the solvent molecules (red) are superimposed on a 3F_o - 2F_c composite annealed omit map contoured at 1.2σ (6% of the model omitted for the calculation). The alternate conformation of residues 1492–1494 is colored in cyan.
in the T/A complex are not observed in the present structure. However, the water molecule mediating the intramolecular hydrogen bond between rings I and II is observed in the two sites, as in the T/A complex. In addition, N1 on ring II binds to a water molecule (in site 1 only). The methyl groups that point away from the RNA might be responsible for a local disturbance of the hydration shell that is observed in the other complexes, therefore accounting for the global decrease of observed solvent molecules bound to the complex.

Crystal packing

The crystal packing contacts involve side-by-side interactions and head-to-tail stacking of helices along the X and Y axis as well as hydrogen bonds involving the bulged adenine bases of each site along the Z axis (Figure 7). The twist angle between the base-pairs at the head-to-tail interface of stacked helices is approximately 90°. Therefore, in this type of pseudo-infinite helix, the adenine bases of site 1 point in the same direction and the adenine bases of site 2 in the opposite direction along the Z axis. Adenine bases 1492 and 1493 of site 1 form, respectively, type II (sugar–sugar cis) and type I (sugar–sugar trans) base interactions by making direct hydrogen bonds to the shallow groove of two stacked G=C pairs located in the middle of a symmetry-related molecule (Supplementary Material). In site 2, A1492 forms a type II interaction to a C=G pair, whereas the tight type I interaction observed with A1493 in site 1 is replaced by H-bonds involving only N1 and N6 of the adenine in the shallow groove of a G=C pair (Supplementary Material). Consequently, the A-minor motif does not occur in site 2 and the intermolecular interactions involving the bulged adenine bases of site 2 are weaker than those involving adenine bases of site 1. This is further sustained by the two conformations that were refined for residues 1492–1494 in site 2. As a result, by relying only on these interactions, the growth of the crystal along the Z direction is likely to be slow,
which may account for the thinness of the crystals observed in that direction (10 µm).

**Discussion**

**Compatibility with the 30 S particle structures**

We developed a system that proved to be successful in the crystallization of three aminoglycosides belonging to distinct sub-groups: paromomycin (a 4,5-2-DOS derivative),\(^8\) tobramycin (a 4,6-2-DOS derivative of the kanamycin sub-group),\(^9\) and geneticin (a 4,6-2-DOS derivative of the gentamicin sub-group). As already observed for the T/A and P/A complexes, the structure of the A site inside the G/A complex is close to the conformation of the A site bound to paromomycin in the 30 S particle.\(^7\) The binding of geneticin therefore induces a similar bulged out conformation for adenine bases 1492 and 1493 (Figure 4(a) and (b); Supplementary Material). Alignment of the sugar-phosphate backbones of the nucleotides forming the A site (RMS deviation = 0.7 Å)
results in RMS differences of 2.4 Å and 1.3 Å between the bulged adenine bases in sites 1 and 2, respectively, of the present structure and those inside the 30S particle in complex with paromomycin, mRNA and tRNA (Supplementary Material).

In addition, the crystal structure of the G/A complex shows various types of base-triple interactions involving A1492, A1493 and the shallow groove of Watson–Crick pairs belonging to symmetry-related fragments. Adenine bases 1492 and 1493 are mobile inside the free ribosome and bulge out in order to bind and recognize the four types of the Watson–Crick pairs formed by the cognate mRNA (codon)–tRNA (anticodon) helix. By binding to the A site, the aminoglycoside paromomycin stabilizes these particular interactions involving the rRNA, tRNA and mRNA, thereby leading to a loss of discrimination between cognate and near-cognate codons. The A-minor motif observed in site 1 of the G/A complex mimics the decoding as it has been inferred from the structure of the 30S particle bound to paromomycin, tRNA and mRNA. The structure of the G/A complex therefore supports the effective binding mode of the aminoglycosides and the presently accepted model for the role of the universally conserved adenine bases in the decoding mechanism.

Comparison with the gentamicin C1a/A site complex solved by NMR

The only other structural information available for aminoglycosides of the gentamicin group binding to the A site comes from an NMR structure of gentamicin C1a complexed to a 27 nucleotide stem-loop containing the A site (PDB identifier: 1BYJ). Compared to geneticin (a 4,6-2-DOS aminoglycoside containing a 6-OH), gentamicin C1a (a 4,6-2-DOS aminoglycoside containing a 6-NH3+) lacks hydroxyl groups O3 and O4, as well as methyl group C7 on ring I (Figure 1). Rings II and III are therefore homologous in the two antibiotics. The conformation of geneticin in the G/A complex is roughly similar to the conformation of gentamicin C1a in the NMR structure (RMS difference based on the ring atoms common to both antibiotics = 0.7 Å) (Supplementary Material). However, the RNA conformations display differences, which cause dissimilarity in the number and type of hydrogen bonds involving the antibiotic and the RNA. In the gentamicin C1a complex, adenine bases 1492 and 1493 do not bulge out from the helix and the U1406·U1495 pair is roughly symmetrical (<distance (N3–N3) > ≈ 4.1 Å and <distance (O4–O4) > ≈ 2.8 Å in the 38 deposited NMR structures). Consequently, RMS deviations between each set of the two bulged adenine bases observed in the G/A complex and the adenine bases of a single representative structure of the gentamicin C1a complex are between 5.4–6.0 Å (Supplementary Material).

In addition, whereas ring I stacks against G1491 in the NMR structure as well, it does not bind to A1408 as observed in the G/A complex. Furthermore, the ammonium N3 on ring II is not fully dehydrated in the gentamicin C1a complex. The four direct hydrogen bonds observed between ring III of geneticin and residues 1405 and 1406 are shorter (2.7–2.9 Å) than the similar contacts observed in the gentamicin C1a complex.
(3.3–4.0 Å). The NMR structure of the gentamicin C1a complex delineated the binding site properly, but by being in agreement with the 30 S particle structures,7,20 the crystal structure of the G/A complex provides a more detailed and accurate description of the binding mode of a gentamicin compound to the eubacterial A site.

Compatibility with results from biochemistry and microbiology

Aminoglycosides bind to the prokaryotic A site with dissociation constants (Kd) in the 1–2 μM range.42,43 Neomycin seems to exhibit more affinity to the A site (Kd = 0.05 μM), probably because of its additional positive charge (Figure 1 and Table 2). Even if no binding data has been obtained for geneticin to date, it can be inferred from the binding measurements previously performed on several closely related aminoglycosides that geneticin would bind with similar affinity to the A site. In addition, the conserved binding mode of the aminoglycosides (more than half of the hydrogen bonds observed in the G/A, T/A and F/A complexes are identical) is consistent with a dissociation constant for geneticin in the micromolar range.

The present work is consistent with the minimal inhibitory concentrations (MICs) measured in vitro for wild-type bacteria and for resistant bacteria bearing mutated A sites.44–48 The MIC values for the wild-type A site are 5, 2.5 and 10 μg/ml for geneticin, tobramycin and paromomycin, respectively.47,48 The MIC value for paromomycin increases to 160 μg/ml for a mutant possessing an A1408G mutation in the 16 S ribosomal RNA, whereas it remains unchanged for geneticin.47,48 The A1408G mutation also causes severe resistance against tobramycin.44,44 This mutation is the only mutation to be found in pathogens having acquired resistance in vivo.44–46 As discussed previously,9 a mutation of A to G would likely disturb the formation of the pseudo-base-pair between ring I of the aminoglycoside and residue 1408. This disruption has a less strong effect on some 4,6-disubstituted aminoglycosides,43 probably because their binding to the A site can rely on the several direct hydrogen bonds made to some 4,6-disubstituted aminoglycosides.47 Noteworthy, the 4,6-disubstituted compounds are less affected by this mutation than the 4,5-disubstituted antibiotics by the A1408G mutation. N1 of ring II of the 4,5-disubstituted paromomycin makes a direct hydrogen bond to O4 of U1495 and O6 of ring II makes a water-mediated hydrogen bond to O4 of U1406 (Figure 4(d)). As previously mentioned,9 the mutation of the U1406-U1495 pair to an A1406-U1495 pair (canonical or not) would probably replace the water-bridged hydrogen bond to O4 of U1406 by a direct hydrogen bond to N6 of A1406. In the 4,6-disubstituted aminoglycosides, O6 is involved in the covalent linkage to ring III and the mutation would cause a clash between adenine 1406 and ring III. Remarkably, in addition to the conserved hydrogen bond to O4 of U1495, geneticin makes a direct hydrogen bond from O4 to O2P of U1406 (Figures 4(d) and 5(c)). Geneticin seems to be slightly more affected by the mutation, probably because the formation of the A1406-U1495 pair would move O2P of residue 1406 too far away to interact with O4 of ring III. As previously suggested on the basis of microbiological data,44–46 the present analysis sustains that resistance is produced in the mutated strains by a decreased affinity of the aminoglycoside to the A site.

Insights into human toxicity

Aminoglycosides are highly toxic to mammals, through kidney and ear-associated illnesses.25,26,40 This toxicity, whose origin is still controversial, probably results from a combination of different mechanisms, depending on the number of positive charges on the antibiotic, on the antibiotic concentration in the cell and on the organ where the toxicity develops. Known mechanisms include: production of hydroxyl radicals by geneticin/metal ion complexes49–51 and disturbance of membrane functionalities by interaction with phospholipids and inhibition of the activity of the phospholipases.26 Geneticin has four positive charges around neutral pH values, compared to five for paromomycin and tobramycin (Table 2). Its electrostatic interactions with the acidic phospholipids might therefore be different from that of aminoglycosides bearing five charges (review26) but its transport through the
Geneticin Bound to the Eubacterial Ribosomal A Site

hydrophobic core of the membrane could be facilitated. The binding of some aminoglycosides like paromomycin and more remarkably geneticin to the eukaryotic A site has also been considered as a possible source of toxicity.\textsuperscript{24,52} Phylogenetic analysis reveals that sequence differences in the A site between prokaryotic and eukaryotic organisms are observed for three nucleotides (rRNA sequences from the Ribosomal Database Project II server\textsuperscript{53}). Whereas prokaryotes universally have an adenine at position 1408, eukaryotes always have a guanine. The 1409–1491 base pair is a C=G for 80% of the prokaryotes (the remaining 20% have an A-U pair), and for 40% of the eukaryotes (the remaining 60% have a C-A pair). In addition to binding to the wild-type prokaryotic A site, geneticin has been shown to bind to the A site of a wide variety of protozoa and parasites.\textsuperscript{20,21,27} Since all these eukaryotic organisms have a G1408 and a C1409=G1491 pair, their A site is similar to the A1408G naturally mutated prokaryotic A site. Therefore, apart from the differences that might occur in the cell uptake and in the antibiotic concentrations inside the cell, we can expect that geneticin is able to interfere with the protein synthesis in these eukaryotic organisms by binding to the A site and producing a similar conformational change.

Interestingly, geneticin also binds to yeast\textsuperscript{22} and higher eukaryotes, which have a G1408 but a C1409-A1491 pair, and to prokaryotes having an A1409-U1491 pair (E. C. Böttger, personal communication). Hence, geneticin seems to be able to accommodate three different types of base-pairs at position 1409–1491 (G=C, C=A, A-U). Whereas paromomycin makes a direct hydrogen bond to N7 of G1491, tobramycin only forms a water-mediated hydrogen bond and no contacts are observed for geneticin. This would imply that different base-pair geometries can be accommodated by geneticin (and less easily or not at all by the other aminoglycosides), as long as these pairs allow stacking against ring I. Stacking of six-membered rings in the chair conformation against aromatic rings has been observed for small molecules.\textsuperscript{54} The type of interaction also occurs in proteins as a hydrophobic patch of the axial C–H bonds of the chair rings to the π-electrons of the carbon atoms forming the aromatic rings of tryptophan or tyrosine residues.\textsuperscript{55,56} The planes of the chair and the aromatic rings are roughly parallel and do not necessarily stagger completely. Therefore, the various arrangements\textsuperscript{57,58} that the different residues at positions 1409 and 1491 could display should not prevent any stacking of ring I. Furthermore, from the observation of the two conformations refined for residues 1492–1494 in site 2 (Figure 6), it appears that the phosphate groups of the bulged adenine bases have enough flexibility to accommodate the slightly different C1′–C1′ distances that could result from different 1409–1491 base-pair types while maintaining the binding to geneticin. The origin of the toxicity of geneticin for a wide range of organisms can thus be partly attributed to its possible binding by a hydrogen bonding pattern allowing the stacking of ring I to any kind of Watson–Crick or non-Watson–Crick pair at position 1409–1491.

**Figure 8.** Geneticin bound to the shallow groove of the U1495-U1406 pair. The view is similar to that shown in Figure 5(c). The interaction is shown in surface representation. Ring I of geneticin is omitted for clarity.

**Strategies for drug design: taking into account the U-U pair**

Besides the importance of the flexibility of the bulge formed by residues 1408, 1492 and 1493 in the A site,\textsuperscript{37} a major role in the function of the ribosome must be played by the highly conserved U1406-U1495 pair inserted between two G=C pairs. The U-U pair displays a particular combination of features like a strong electro-negative deep groove (two O4 atoms), a shorter C1′–C1′ distance and some flexibility in the spatial arrangement of the two pyrimidine rings (Figure 8). It is noteworthy that no other base-pair is either strictly isosteric or has the same electrostatic pattern than the U-U pair, a fact which may account for its conservation. In the G/A complex as in all the crystal structures containing the A site solved to date,\textsuperscript{7–9,37,40} the U-U pair adopts a conformation where O4 of U1406 makes a direct hydrogen bond to N3 of U1495. The conformation is stabilized in the T/A\textsuperscript{8} and P/A\textsuperscript{6} complexes by a direct hydrogen bond from N3 of U1406 to O2P of U1495 (Figure 4(d)). The water-mediated hydrogen bond is not observed in the G/A complex, but a direct contact is observed to O2P of U1406. Comparisons with other structures show that cis Watson–Crick/Watson–Crick U-U pairs present two main base-to-base geometries either symmetric\textsuperscript{69,60} or bifurcated.\textsuperscript{61,62} This flexibility allows a close binding of 4,6-disubstituted antibiotics to nucleotides that are located on both sides of the U-U pair (residues 1408 and 1409), as shown from the crystal structures and the mutation.
data. Furthermore, the various types of U-U pairs present different C1′–C1′ distances (between 9.8 Å and 10.6 Å) that can lead to additional accommodation and contacts that stabilize the conformation of the A site bound to the aminoglycoside, as shown with gentamicin.

One design approach should be aimed at compounds that would both stabilize the shifted conformation of the U1406-U1495 pair while interacting with residues on both sides and induce similar conformations for residues 1408, 1492 and 1493. The structure of the G/A complex shows similar conformations for residues 1408, 1492 and 1493. The structure of the G/A complex shows similar conformations for residues 1408, 1492 and 1493. In addition, geneticin seems to be able to accommodate different base-pairs at position 1409–1491. The particular polyanvil character exhibited by geneticin could therefore be of help for the design of compounds that bind to the prokaryotic A site, regardless of the nature of the nucleotides at positions 1408, 1409 and 1491.

Conclusion

The neamine part of the aminoglycoside genetin binds to the A site as observed for related aminoglycosides complexed with the 30 S particle or with an RNA model fragment containing the decoding A site. Compared to the other aminoglycosides of the 4,6-DOS sub-class still prescribed today for strong infections (tobramycin, amikacin and the gentamicins), genetin possesses distinctively positioned functional groups that offer the ability to accommodate several point mutations associated to resistance or to phylogenetic variations. Therefore, by being remarkably not selective for prokaryotes, genetin is a good model to study the toxicity associated with aminoglycosides at the ribosomal level. The comparison of the present structure with the complex of the 30 S particle and of the eubacterial A site bound to paromomycin and tobramycin reveals the important role played by the conserved U1406-U1495 pair in addition to adenine bases 1408, 1492 and 1493. Thus, the non-Watson–Crick U-U pair should be taken into consideration in the design of new antibacterial molecules.

Material and Methods

RNA purification and crystallization

The oligoribonucleotides used previously to crystallize other aminoglycoside/A site complexes were ordered from Dharmacon Research, Inc. (Boulder, CO, USA). Deprotected RNA oligonucleotides were purified to crystallization quality by denaturing gel electrophoresis (20%(w/v) acrylamide/bisacrylamide (19:1), 8 M urea) using TBE buffer (9 mM Tris-borate (pH 8.3), 0.2 mM EDTA) at 50–55°C. The oligoribonucleotides were recovered by the “crush and soak” method at 4°C in 10 mM Tris (pH 7.5), 200 mM NaCl, 0.5 mM Na EDTA during 48 hours with one buffer exchange, followed by ethanol precipitation. The pellets were washed with 95%(v/v) ethanol, evaporated to dryness and resuspended in MilliQ water.

A 2 mM RNA solution containing 25 mM NaCl, 10 mM MgSO4 in 100 mM sodium cacodylate buffer (pH 6.4) was first heated at 85°C for two minutes then slow cooled for two hours 30 minutes to 37°C. One volume of a 4 mM genetin disulfate (Fluka, reference 48753) solution (in 25 mM NaCl, 10 mM MgSO4, 50 mM sodium cacodylate buffer (pH 6.4)) was added to an equal volume of the RNA solution at 37°C. Slow cooling then proceeded to 21°C. Then 1 μl of RNA/genetin solution plus 1 μl of crystallization solution (1.5%–3.5% MPD, 1.0–2.0%(v/v) glycerol, 2 mM genetin, 150 mM KCl, 50 mM sodium cacodylate (pH 6.4)) were equilibrated against 500 μl of 40% MPD by vapor diffusion using the hanging drop method. Thin long and narrow plates (700 μm x 100 μm x 10 μm) organized in clusters appeared after three days at 37°C for the sequence containing a 5’ C overhang.

Data collection, structure determination and refinement

Crystals were equilibrated for 20 minutes in cryoprotecting solutions containing all the components present in the crystallization solution (but with 60–70% MPD and 10% glycerol) before being flash-cooled in liquid ethane (110 K). Data collection occurred on the Joint Structural Biology Beamline ID14-EH4 at the European Synchrotron Radiation Facility (ESRF; Grenoble, France). A 95.5% complete but of poor quality data set (resolution, 2.35 Å; R free = 9.5% (R free = 2.64–2.55 Å) = 32.0%, (I/σ) = 8.0) was obtained by adding 185° collected on one crystal (set 1) to 65° and 28° collected on two other crystals. The reflections were indexed, scaled and converted using the software programs DENZO, SCALEPACK and the CCP4 suite. A correct solution (F corr = 40.3% and R-factor = 54.3%) was found by the molecular replacement program AMoRe in the resolution range 12.0–3.0 Å, using the coordinates of the RNA from. One round of torsion simulated annealing (starting at 1500 K and slow cooling at 50 K/minute; wa = 5.7) in the resolution range 10–2.55 Å in CNS version 1.0 refined the molecular replacement solution from R = 43.0%/R free = 44.7% to R = 34.8%/R free = 38.8% (7% of the reflections were set aside for the R free calculations). 85° of data set 1 collected on beamline ID14-EH4 were added to 100° collected later on another crystal on beamline ID14-EH1 at the ESRF. After data processing, the resolution and the parameters were improved, while the completeness got lower (Table 1). A total of 5% of the reflections were used for the R free calculations in CNS version 1.1. The parameter and topology files for the genetin molecules were obtained on the XDICT server. Occupancy and B-factors were fixed to 1.0 A for 20.0 A, respectively. After one run of torsion simulated annealing (starting at 20.0 K and slow cooling at 25 K/minute; wa = 2.8) on the resolution range 25.0–2.4 Å (cutoff for the rejection of reflections, 1.5) followed by group B-factor refinement
on a 6.0–2.4 Å resolution range, R and \( R_{\text{free}} \) decreased to 32.8% and 33.8%, respectively. Alternating the crystallographic refinement task files in CNS (anneal, refine, minimize, bindividual, water_pick, optimize_wa) using base planarity and Watson–Crick base-pairing restraints, and the manual rebuilding of the structure in the calculated \( \sigma^{a} \)-weighted \( F_{o} \) – \( F_{c} \) and \( F_{o} \) – \( F_{c} \) electron density maps using O\textsuperscript{e} version 8.0.5 improved the model to \( R = 23.1\% / R_{\text{free}} = 26.0\% \). From this stage, the statistics obtained from the task file model.stats were regularly checked to improve the model.

As in the paromomycin complex, the density is missing for the 5’ overhang cytosine. The density in site 1 is better defined than the density in site 2, as observed in the two previous structures.\(^{6,7} \) The experimental electron density map in site 2 could be fit by refining two alternate conformations for the bulged adenine bases A16 and A17, guanine G18 and water molecule W2. After addition of seven water molecules, the model was refined by minimization procedures and Cartesian simulated annealing (starting at 1000 K and slow cooling at 25 K/minute; \( wa = 4.2 \); all residues fixed except for A16-G18 and solvent molecule W2) to reach \( R = 22.5\% / R_{\text{free}} = 25.3\% \). The next improvements consisted in increasing the weight on the constraints for the planarity of the bases forming the U-U pair in site 2 and refining the occupancy factors of water molecules W8 and W21 using the individual task file. Group occupancy refinement was checked for the residues in alternate conformations using the qgroup task file, but the resulting \( q \) factors lying between 0.43 and 0.58 led us to leave them unchanged at 0.5. The alternate conformations observed for W2 were replaced by two distinct water molecules having occupancies of 0.50 (W2 and W40). In addition, the occupancy factors of W34 and W39 were set to 0.5. Finally, multiple rounds of occupancy and B-factor refinement were performed only with solvent molecules W2, W34, W39 and W40. The final structure has \( R = 22.4\% / R_{\text{free}} = 25.0\% \) and contains 965 RNA atoms, 68 antibiotic atoms and 40 solvent molecules.

Data Bank Accession codes

Coordinates and structure factors were deposited in the Nucleic Acid Database (NDB code DR0008) and the RCSB Protein Data Bank (ID code 1MWL).

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