Analysis of the Contribution of Individual Substituents in 4,6-Aminoglycoside–Ribosome Interaction

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Received 12 May 2005/Returned for modification 2 August 2005/Accepted 12 September 2005

The 4,6-disubstituted 2-deoxystreptamines interfere with protein biosynthesis by specifically targeting the ribosomal A site. These drugs show subtle variations in the chemical groups of rings I, II, and III. In the present study we used site-directed mutagenesis to generate mutant strains of Mycobacterium smegmatis mc²155 SMR5 ΔrrnA with mutations in its single rRNA allele, rrnA. This genetic procedure gives rise to strains carrying homogeneous populations of mutant ribosomes and was used to study the contribution of individual chemical substituents to the binding of 4,6-disubstituted aminoglycosides. X-ray crystal structures of geneticin and tobramycin complexed to oligonucleotides containing the minimal bacterial ribosomal A site were used for interpretation of MICs determined for a panel of 4,6-aminoglycosides, including tobramycin, kanamycin A, kanamycin B, amikacin, and gentamicin. Surprisingly, the considerable differences present within ring III did not seem to alter the interaction of the drug with the ribosome, as determined by site-directed mutagenesis of the A site. In contrast, subtle variations in ring I significantly influenced binding: (i) a 4'-hydroxyl moiety participates in the proper drug target interaction; and (ii) a 2'-amino group contributes an additional positive charge to ring I, making the drug less susceptible to any kind of sequence alteration within the decoding site, most notably, to conformational changes induced by transversion of U1495 to 1495A. The 4-amino-2-hydroxyl-1-oxobutyl extension at position 1 of ring II of amikacin provides an additional anchor and renders amikacin less dependent on the structural conformation of nucleotide U1406 compared to the dependencies of other kanamycins. Overall, the set of interactions forming the complex between drug substituents and nucleotides of the A site constitutes a network in which the interactions can partly compensate for each other when they are disrupted.

Most aminoglycosides which are used as broad-spectrum antibacterial agents are representatives of the 4,6-disubstituted 2-deoxystreptamines, e.g., tobramycin, kanamycin, amikacin, and gentamicin (3, 5, 10). The two sugars glycosidically linked to the 4 and 6 positions of the central aminocyclitol ring (2-deoxystreptamine) are responsible for the sequence-specific binding to the highly conserved decoding center within the ribosomal A site (8, 11, 13, 14). Aminoglycosides promote the improper selection of aminocyclitol-tRNA and inhibit the translation of the RNA-mRNA complex (2, 9). Drug-induced miscoding is thought to lead to the synthesis of nonfunctional, misfolded proteins and to cell death (4, 6).

A narrow drug pocket that is able to accommodate a framework of hydrogen bond donors and acceptors is provided by the wobble base pair U1495·U1406; bases A1408, A1492, and A1493; and the base pair C1409-G1449 (Escherichia coli numbering is used throughout the paper) of the 16S rRNA-decoding site (Fig. 1). A large and diverse population of about 50 aminoglycoside-modifying enzymes exists; and these transfer acetyl, phosphoryl, and adenyl groups in a cofactor-dependent manner to virtually every amino or hydroxyl position, thus conferring resistance (3, 5, 12). The 2'- and 6'-NH₃⁺ groups of ring I as well as the 3 position of ring II become acetylated by acetyl coenzyme A-dependent N-acetyltransferases (5). The introduction of an acetyl group at 6'-NH₃⁺ disrupts the hydrogen bond contact between the 6'-NH₃⁺ moiety and A1408 and results in high-level drug resistance (20, 21). Modification of the 2'-NH₃⁺ group most likely influences the structural conformation of the 4,6-disubstituted aminoglycosides by introducing a bulky side chain that interferes with the appropriate positioning of rings II and III (20). The amino group at position 3 of ring II makes hydrogen-bonded contacts to N-7 of G1494 and to the phosphate groups of A1493 or G1494 (20, 21), acetylation of 3-NH₂ would prevent the formation of these interactions. In the case of phosphorylation or adenylating of the OH group at the 2° position, the hydrogen bond to O-6 of G1405 and the neighboring one between N-3° and N-7 of G1405 cannot form (20, 21). A phosphate moiety at the 4° position of ring I would prevent the insertion of ring I into the A site and direct H bonding between O-4° and the phosphate oxygen O-2-P of A1493 (13).

In response to these resistance mechanisms, a large variety of natural and semisynthetic aminoglycoside derivatives have been synthesized. Most studies of the structure-activity relationships of 4,6-disubstituted 2-deoxystreptamines have been carried out with wild-type ribosomes or with heterogeneous populations of mutant and wild-type ribosomes (1, 4, 7, 18). However, this yielded only limited information on the network of hydrogen bond interactions and the van der Waals and elec-
trostatic contacts that maintain the drug-target complex. With the introduction of microorganisms carrying homogeneous populations of mutated ribosomes, the roles of different substituents in drug binding can now be characterized at the molecular level. In such an in vivo assay system, the drug susceptibility phenotype conferred by a specific mutation is likely to reflect the importance of a specific interaction (13–16). In this study, we have used site-directed mutagenesis of the drug binding site to construct recombinant microorganisms that carry homogeneous populations of mutant ribosomes. In particular, we determined the susceptibilities of various mutant strains to kanamycin A, kanamycin B, and amikacin and compared the results to previously published data to characterize the contributions of subtle variations in the chemical moieties of rings I, II and III of 4,6-disubstituted 2-deoxystreptamines to drug binding.

MATERIALS AND METHODS

Bacterial strains. The strains of Mycobacterium smegmatis mc²155 SMR5 ΔrrmB (13, 14) listed in Table 1 were cultured on Luria-Bertani (LB) agar plates. Mutant strains of M. smegmatis mc²155 SMR5 ΔrrmB were obtained by selective plating or by the introduction of point mutations by using RecA-mediated homologous recombination, as described previously (13, 14, 17).

Determination of MICs. Strains of M. smegmatis mc²155 SMR5 ΔrrmB (13, 14) were cultured on LB agar plates. Single-colony cultures, which were grown in liquid LB medium supplemented with 0.05% Tween 80, were used for MIC assays in a microtiter plate format, as described previously (13, 14). In brief, the antibiotics to be tested were added to 200 µl of starting cultures with an optical density at 600 nm of 0.025 in a twofold series of dilutions with concentrations...
### RESULTS

Derivatives of the *M. smegmatis* mc^\text{155}^ SMR5 ΔrnmB with single point mutations in the 16S rRNA were obtained by RecA-mediated gene conversion. Double mutations were introduced by selective plating by starting with strains with a single point mutation (13, 14). A large set of strains carrying mutations in key nucleotides of the binding pocket were generated (Fig. 2 and Table 1).

The 4,6-disubstituted 2-deoxystreptamines share close structural similarities. Two amino sugar moieties are connected to a central aminocyclitol ring (2-deoxystreptamine; ring I), one each at positions 4 and 6. The members of this antibiotic family can be distinguished by subtle variations within rings I, II, and III. The chemical groups linked to ring III allow the separation of the compounds into two subclasses: the kanamycins and the gentamicins. Compared to the kanamycins (which encompasses tobramycin, kanamycin B, kanamycin A, and amikacin), ring III of the gentamicins (which encompasses gentamicin, and geneticin) lacks a methoxy group at position 5°. Instead, ring III of the gentamicin subclass has two additional methyl group substitutions at positions 4° and 3° (Fig. 3 and 4).

**Kanamycins.** The drug susceptibility measurements given in Table 2 show that the compounds of the kanamycin subclass exhibit very similar potencies against wild-type *M. smegmatis* mc^\text{155}^ SMR5 ΔrnmB (MICs, 2 to 4 μM), irrespective of the differences in their chemical structures. In general, mutations within the ribosomal A site affect the relative activities of the compounds similarly. However, a close inspection of the MIC data reveals subtle differences, as discussed below.

Tobramycin and kanamycin B are chemically identical, with the exception that the latter has a hydroxyl group at position 3° of ring I (Fig. 3). Removal of a hydroxyl group proximal to an amino group leads to an increased basicity of the amino group and, thus, a higher positive charge at a given pH (19). According to the MIC data, both drugs are similarly affected by mutations within the A site. However, compared to tobramycin, the activity of kanamycin B seems to rely more on the type of nucleotide at 16S rRNA position 1409, i.e., C versus G or U (Table 2). Thus, the presence or absence of a 3°-hydroxyl group seems to be relevant mainly for the interaction with base pair C1409-G1491, which closes the binding pocket at the lower stem. In the crystal structures, geneticin (21) presents a direct H bond between the O-3° and O-2-P of A1492 while, in tobramycin (20), there is an extensive network of water molecules linking N-2° and N-7 of G1491, O-2-P of A1492, and O-1-P of A1493.

Kanamycin A is distinguished from kanamycin B by the replacement of the NH\textsubscript{3}^+ group at position 2° of ring I with a hydroxyl group, which leads to the loss of a positive charge. As seen in Table 2, the exchange of the NH\textsubscript{3}^+ group for an OH group makes the drug more susceptible to any kind of sequence alteration within the decoding site, in particular, the transversion of U1495 to 1495A.

Amikacin is a derivative of kanamycin A which possesses an additional 4-amino-2-hydroxyl-1-oxobutyl at position 1 of the 2-deoxystreptamine ring that extends on the side of A1495. This extension allows amikacin to partly compensate for the unfavorable geometry provoked by mutations 1406A and 1406C (Table 2). However, introduction of the Watson-Crick base pair 1495A-U1406 and of the wobble base pair 1495A·U1406C (Table 2). Thus, the presence or absence of a nucleotide at 16S rRNA position 1409, i.e., C versus G or U (Table 2). Therefore, the exchange of the NH\textsubscript{3}^+ group for an OH group makes the drug more susceptible to any kind of sequence alteration within the decoding site, in particular, the transversion of U1495 to 1495A.
G1491 or C1409 conferred significantly less resistance to amikacin than to kanamycin A.

**Gentamicins.** Gentamicin inhibits protein synthesis in wild-type *M. smegmatis* mc²155 SMR5 ∆mrr to a similar extent as the kanamycins. However, the MICs determined for the mutants reveal significant differences between those of gentamicin and the kanamycins (Table 2). In particular, as revealed by the MIC data for mutant 1406A, gentamicin is much more susceptible than the kanamycins to alterations of U1406 (Table 2). Interestingly, alteration of U1495 affects both gentamicin and kanamycin similarly.

Except for the 6'H group, ring I of geneticin is identical to that of kanamycin B; rings II and III are conserved in the gentamicin subclass. Of all 4,6-disubstituted 2-deoxystreptamines tested, geneticin has the lowest activity against wild-type *M. smegmatis* and the greatest dependence on the stacking interaction with the base pair C1409-G1491. This finding can be correlated to the unique hydroxyl group at position 6' of ring I of geneticin (13, 14). Because of the low affinity of geneticin to the A site of the small ribosomal subunit, a quantitative estimation of resistance levels conferred by changes in the wobble base pair U1495 · U1406 is not possible at present.

**DISCUSSION**

Point mutations within the decoding site of the small ribosomal subunit influence the binding of aminoglycosides to various extents. Use of *M. smegmatis* mc²155 SMR5 ∆mrr with mutations in its single *rrn* allele (*rrnA*) allowed for the construction of strains carrying homogeneous populations of mutated ribosomes, thus facilitating the correlation of ribosomal drug susceptibility with the distinct chemical properties of the antibiotics.

Compared to an in vitro system with isolated ribosomes, in vivo susceptibility testing of whole cells can provide only limited information with respect to the efficiency of a drug against wild-type ribosomes, as the MICs of different drugs are also influenced by drug uptake or export across the bacterial cell wall. In our system, kanamycin A and gentamicin are equally potent and are more potent than tobramycin and kanamycin B, which are two to four times less active than kanamycin A and gentamicin. In an in vitro system of R17 phage RNA-directed polypeptide synthesis, kanamycin B, tobramycin, and gentamicin inhibit the ribosome almost twice as efficiently as kanamycin A (1). However, the in vivo model system used here provides valuable information on the ribosomal resistance conferred by mutational alterations of the decoding site. This is possible because the strains used are isogenic, and variability...
in the uptake of any given aminoglycoside is excluded as a
determinant by calculation of relative levels of resistance.

The wobble base pair U1495 · U1406 closes the upper part
of the drug binding pocket. All 4,6-disubstituted aminoglyco-
sides form a single direct contact to this wobble base pair via
a hydrogen bond between N-1 of ring II and O-4 of U1495
(Fig. 1) (20). Transversion of U1495 to an adenine alters the
geometry of the base pair such that appropriate positioning of
its alteration and are unable to place ring III in an ap-
propriate position. The nonisosteric pair 1495A-1406C and the
nonisosteric pair 1495A-1406C provoke high levels of resistance, pre-
sumably due to major changes in the base pair geometry (14).

![Diagram of Genecin and Gentamicin](image)

FIG. 4. Resistance profile of the 4,6-disubstituted 2-deoxystreptamines (gentamicin subclass) represented as comparison graphs. Each axis of
the comparison graph represents the influence of the corresponding mutation introduced (indicated in gray). The relative resistances conferred
by each of the mutations are plotted on a log scale, with the smallest value in the innermost circle. Points lying on the outermost circle indicate
that the corresponding mutations result in relative resistance outside the measurable range. Geneticin binds weakly to the wild-type ribosomes;
thus, quantitative analysis is limited; to reflect this limitation, the values outside the measurable range are depicted by hatched areas.

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<th>Tobramycin</th>
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<th>Kanamycin A</th>
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<td></td>
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**TABLE 2. Drug susceptibilities of rRNA mutants**

*a* The substitution modes of the compounds in the kanamycin subclass, corresponding to the chemical representation of the drugs in Fig. 3, are as follows: for tobramycin, R₁ = NH₄⁺; R₂ = H; R₃ = H; for kanamycin B, R₁ = NH₄⁺; R₂ = OH; R₃ = H; for kanamycin A, R₁ = OH; R₂ = OH; R₃ = H; for amikacin, R₁ = OH; R₂ = NH₂; R₃ = C₆H₅NO₂.

*b* The substitution modes of the compounds in the gentamicin subclass, corresponding to the chemical representation of the drugs in Fig. 4, are as follows: for genecin, R₁ = OH; R₂ = OH; R₃ = CH₃; R₄ = OH; for gentamicin, R₁ = H; R₂ = H; R₃ = CH₃; R₄ = NH₄⁺; NH₂CH₃.

*d* RR, relative resistance, calculated by dividing the MIC for the mutant by the MIC for the wild type.

With the exception of the 1406C-1495G mutant, the MIC data for tobramycin, genecin, and gentamicin have been published elsewhere (13, 14) and are included here for comparison.
Our observations suggest that the methylation pattern of ring III characteristic of the gentamicin subclass does not significantly contribute to drug binding; this interpretation is supported by structural data, which show that the methyl groups point away from the decoding pocket (20, 21). The exceptional steric positioning of the 4′-OH group of the gentamicins allows these aminoglycosides to form an additional hydrogen bond between ring III and one of the phosphate oxygens of U1406. It is thought that the stabilizing effect of the hydrogen bond contact with residue U1406 is important for the drug-ribosome interaction (Fig. 1) (21); this is also reflected in the high-level drug resistance conferred by the 1406A alteration (Table 2).

The extent to which a drug is dependent on proper U1495·U1406 conformation may be correlated with the chemical groups linked to ring I. Kanamycin B possesses a 3′,4′-dihydroxy glucopyranosyl, whereas the otherwise identical compound tobramycin has a 4′-hydroxy ring I. Both aminoglycosides are equally dependent on the correct conformation of the U1495·U1406 pair. Hence, the possible hydrogen bond between the 3′-OH group and the phosphate of A1492, as postulated in the crystal structures of paromomycin and gentamicin (22, 21), may not contribute greatly to the activities of the aminoglycosides. Based on findings from in vitro assays, it was suggested that the presence or the absence of the 4′-hydroxyl group should not markedly affect the biological activities of the 4′,6-disubstituted aminoglycosides (1). However, our data reveal that binding of the 3′,4′-dideoxy gentamicin is more susceptible to mutational alteration of U1406 than the 4′-hydroxy aminoglycosides tobramycin and kanamycin B.

The exquisite susceptibility of gentamicin to the 1406A alteration thus involves at least two possible contacts: (i) a lack of hydrogen bonding between the 4′-OH moiety and the phosphate group of A1493, and (ii) hydrogen bonding between the 4′-OH group and the phosphate oxygen of U1406. At present it is not possible to define the contribution of each of these interactions to the drug resistance conferred by 1406A ribosomes. Apparently, the lack of these hydrogen-bonding interactions does not allow gentamicin to compensate for the unfavorable positioning of rings II and III as a result of mutational alterations of U1406.

The 6′-amino kanamycin, kanamycin A, is about fourfold more sensitive to the transversion mutation of U1495 and U1406 than the 2′,6′-diamino kanamycin, kanamycin B. The available crystal structures propose the involvement of the 2′-amino group in water-mediated hydrogen bonds with O-5 of ring II in both tobramycin (20) and gentamicin (21) and with the phosphate group of A1492 in tobramycin (Fig. 1). The importance of the 2′-NH₂⁺ group for aminoglycoside binding observed in our studies is further underlined by the results obtained with an in vitro system which compared the activities of enzymatically modified 2′,6′-NH₂⁺-kanamycin B and 2′-OH, 6′-NH₂⁺-kanamycin A against wild-type ribosomes. Kanamycin A is completely inactivated by N-acetyltransferase-mediated N-acetylation of the 6′ position, while the identically modified kanamycin B still has almost the same activity as the 2′-NH₂⁺,6′-OH-kanamycin C (1).

Stacking of ring I on G1491 is required for the correct intercalation of the aminoglycosides into the target site. However, 4,6-disubstituted 2-deoxystreptamines seem to be less dependent than 4,5-disubstituted aminoglycosides on the correct conformational structure of the base pair C1490-G1491 (13). This might be due to the synergistic effects conferred by different properties of the 4,6-disubstituted 2-deoxystreptamines, such as the positioning of ring III, the possibility of the formation of an H bond between G1405 and N³ of ring III, and the lack of additional interactions with G1491 (13).

Chemically, amikacin has a ring I identical to that of kanamycin A. In contrast to kanamycin A, however, alterations of U1406 do not confer resistance. Apparently, the 4-amino-2-hydroxyl-1-oxobutyl extension at position 1 of ring II of amikacin provides an additional contact. Amikacin is similarly affected by transition of nucleotide U1495 like all other kanamycins, making it difficult to predict with which residues the 4-amino-2-hydroxyl-1-oxobutyl might interact.

Our data indicate that the patterns of resistance conferred by mutations within the ribosomal A site show general features but may also be dependent in part on the different chemical substitutions of the various 4,6-disubstituted 2-deoxystreptamines. Our data not only validate the available X-ray crystallography data but also provide information on the importance of each binding contact in the drug-ribosome interaction.

ACKNOWLEDGMENTS

This study was supported by grants from the Swiss National Science Foundation to E.C.B. (grant 3200-BO-100780, NRP 49).

We thank Andrea Vasella for helpful comments on the manuscript, Tanja Janusic for expert technical assistance, Alice Makovec for typing the manuscript, and Boris François for kindly providing Fig. 1.

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