Basis for prokaryotic specificity of action of aminoglycoside antibiotics

Michael I. Recht, Stephen Douthwaite and Joseph D. Puglisi

Department of Structural Biology, Stanford University School of Medicine, Stanford, CA 94305-5126, USA and Department of Molecular Biology, Odense University, Campusvej 55, DK-5320, Odense M, Denmark

© European Molecular Biology Organization 3133

Introduction

The aminoglycosides (Figure 1) are related bactericidal antibiotics that bind directly to prokaryotic 16S rRNA at the decoding region A site (Figure 2) (Moazed and Noller, 1987; Woodcock et al., 1991), inducing codon misreading and inhibiting translocation both in vitro (Davies et al., 1965) and in vivo (Edelmann and Gallant, 1977). The utility of aminoglycosides as antibacterial agents arises from their specificity of action. Although they target a conserved region of rRNA sequence, they discriminate between prokaryotic and eukaryotic ribosomes. Prokaryotic and mitochondrial ribosomes, which are sensitive to the action of many aminoglycosides, have an adenosine at position 1408 of the 16S rRNA (Gutell, 1994) (numbered according to the Escherichia coli sequence). In contrast, eukaryotic cytoplasmic ribosomes, which are insensitive to most aminoglycosides (Wilhelm et al., 1978), have a guanosine at position 1408 (Gutell, 1994) (Figure 2).

The difference in potency of aminoglycoside antibiotics could be caused by a variety of factors. Although the sensitivity of prokaryotic organisms to the aminoglycosides could be due to differences in antibiotic uptake and membrane permeability between prokaryotic and eukaryotic cells, prokaryotic ribosomes are inhibited in vitro at a lower antibiotic concentration than eukaryotic ribosomes (Wilhelm et al., 1978). Nonetheless, there are numerous differences between the ribosomes of prokaryotes and eukaryotes. In addition to the identity of nucleotide 1408, eukaryotic ribosomes differ from bacterial ribosomes in both the rRNA and protein components. It is not known whether eukaryotic resistance to certain aminoglycosides is due to lower affinity of the antibiotics for the ribosome or to non-productive binding of the antibiotics to the eukaryotic ribosome.

Biochemical and structural studies using a model oligonucleotide of the decoding region (Fourmy et al., 1996; Recht et al., 1996) demonstrated the importance of an A1408-A1493 base pair for the high-affinity binding of the antibiotic paromomycin to the decoding region. The geometry of the A·A pair creates the specific binding pocket for the critical ring I of aminoglycosides (Fourmy et al., 1996; Yoshizawa et al., 1998). Despite our structural studies, it is critical to demonstrate the link between binding affinity and antibiotic action in vivo. To understand whether the change from A1408 to G in eukaryotes is the origin of specificity of aminoglycosides for prokaryotes, we have introduced the G1408 mutation in E. coli ribosomes. Here we show that expression of 16S rRNA containing an A1408 to G mutation in E. coli strain DH1 confers resistance to many aminoglycoside antibiotics. The results explain the prokaryotic specificity of action for the aminoglycosides.

Results

Growth phenotype of cells expressing mutant 16S rRNA

Expression of 1408G 16S rRNA in E. coli DH1 in the absence of aminoglycoside antibiotic has no apparent growth phenotype (Figure 3). Since the plasmid-encoded rRNA comprises only 60% of the total rRNA (Recht et al., 1999), phenotypic effects of the 1408G mutation could be masked by the presence of 1408A wild-type ribosomes. The antibiotic spectinomycin inhibits translocation of genome-encoded ribosomes (Gale et al., 1981), but expression of plasmid-encoded 16S rRNA containing a C1192 to U mutation supports growth in media containing spectinomycin (Sigmund et al., 1988). Therefore, in the presence of spectinomycin, all actively translating ribosomes are of plasmid-encoded origin. Cells expressing
Fig. 1. Chemical structures of 2-deoxystreptamine (ring II)-containing aminoglycosides, all of which bind to the decoding region A site. Neamine consists of rings I and II of neomycin. The 4,5-disubstituted (neomycin, paromomycin) and the 4,6-disubstituted (gentamicin, kanamycin) are the two general classes of 2-deoxystreptamine-containing aminoglycosides.

Fig. 2. Sequence of the decoding region A site from prokaryotic (E.coli) and eukaryotic (Tetrahymena thermophila and Homo sapiens) rRNAs. Sequences are numbered according to the E.coli sequence. Nucleotides that are universally conserved in all non-mitochondrial 16S-like rRNAs are shown in green (Gutell, 1994). Nucleotides that are conserved in all eukaryotic cytoplasmic ribosomes are indicated in red. The 1408G mutation was introduced into the 16S rRNA sequence and expressed from plasmid pKK3535 (Recht et al., 1999).

1408G 16S rRNA containing the additional 1192U mutation were able to grow, with a slightly slower growth rate than otherwise wild-type ribosomes (83 versus 59 min doubling time), in media containing 50 µg/ml spectinomycin, indicating that these mutant ribosomes are functional (Figure 3).
Expression of the 1408G 16S rRNA, with cells grown in media containing 50 µg/ml spectinomycin, conferred >200-fold resistance to the aminoglycosides kanamycin A, amikacin and apramycin (Table I). In addition, the mutation conferred >64-fold resistance to neomycin, gentamicin C, tobramycin and neamine. Only 4-fold resistance was observed for paromomycin, and there was no resistance conferred to G418. No resistance to streptomycin, a structurally distinct aminoglycoside, was observed.

Resistance was not due to altered expression of the mutant 16S rRNA, as cells grown in the presence of an aminoglycoside did not show an increased proportion of 1408G rRNA. The cells had a slower growth rate in the presence of high concentrations of any aminoglycoside, and growth was not stimulated in the presence of sub-lethal concentrations of the antibiotic. Addition of neomycin to 20 µg/ml to a culture expressing 1408G 16S rRNA caused an increase in doubling time, from 83 to 245 min. Cells propagated in liquid culture in the presence of 40 µg/ml to a culture expressing 1408G 16S rRNA caused an increase in doubling time, from 83 to 245 min. Cells propagated in liquid culture in the presence of 40 µg/ml to a culture expressing 1408G 16S rRNA caused an increase in doubling time, from 83 to 245 min.

Growth of cells in the presence of ampicillin instead of spectinomycin, with or without the additional mutation at C1192, caused a similar, but less dramatic, resistance phenotype (4- to 32-fold resistance). In the presence of spectinomycin, ribosomes containing the additional 1192U mutation comprise 75–80% of the actively translating ribosomes (polysome fraction) (Johanson and Hughes, 1995; data not shown) compared with 60–65% in the presence of ampicillin (Recht et al., 1999). Therefore, the addition of spectinomycin enhances the resistance phenotype of the 1408G mutation by removing the aminoglycoside-sensitive genome-encoded ribosomes from the pool of actively translating ribosomes.

**Table I. Minimum inhibitory concentration of various aminoglycosides against E.coli DH1 expressing wild-type or 1408G 16S rRNA**

<table>
<thead>
<tr>
<th>Aminoglycoside</th>
<th>Wild-type</th>
<th>1408G</th>
<th>Relative resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kanamycin A</td>
<td>2.5 µg/ml</td>
<td>1280 µM</td>
<td>512</td>
</tr>
<tr>
<td>Amikacin</td>
<td>1.25 µg/ml</td>
<td>320 µM</td>
<td>256</td>
</tr>
<tr>
<td>Apramycin</td>
<td>5 µg/ml</td>
<td>&gt;1280 µM</td>
<td>&gt;256</td>
</tr>
<tr>
<td>Neomycin</td>
<td>5 µg/ml</td>
<td>640 µM</td>
<td>128</td>
</tr>
<tr>
<td>Gentamicin C</td>
<td>1.25 µg/ml</td>
<td>160 µM</td>
<td>128</td>
</tr>
<tr>
<td>Tobramycin</td>
<td>1.25 µg/ml</td>
<td>160 µM</td>
<td>128</td>
</tr>
<tr>
<td>Neamine</td>
<td>20 µg/ml</td>
<td>&gt;1280 µM</td>
<td>&gt;64</td>
</tr>
<tr>
<td>Paromomycin</td>
<td>5 µg/ml</td>
<td>20 µM</td>
<td>4</td>
</tr>
<tr>
<td>G418</td>
<td>2.5 µg/ml</td>
<td>2.5 µM</td>
<td>1</td>
</tr>
</tbody>
</table>

The MIC is the minimum concentration of antibiotic that fully inhibited growth after 24 h incubation at 37°C. Relative resistance is the value of the MIC against cells expressing 1408G (priming site V, 1192U) 16S rRNA divided by the MIC against cells expressing wild-type (priming site V, 1192U) 16S rRNA.

**Binding of aminoglycosides to 1408G ribosomes**

Aminoglycoside resistance could result from either decreased affinity of the antibiotic for the ribosome or non-productive binding of the drug to the ribosome. To determine if there was a correlation between resistance and low-affinity binding of the aminoglycoside to the 30S subunit, chemical footprinting experiments were performed on 1408G 30S subunits in the presence of increasing concentrations of neomycin. The mutant ribosomes in the mixture could be monitored exclusively by primer extension due to the presence of priming site V (Powers and Noller, 1993), a silent mutation present only in the plasmid-encoded 16S rRNA. Even with 1 mM neomycin present, only a slight footprint is observed at G1494(N7) (Figure 5A). In contrast, when the wild-type neomycin-sensitive 30S subunits in the same reaction are monitored (Figure 5B), neomycin causes a strong footprint at G1494(N7) and A1408(N1) at 10 µM antibiotic.

The cells expressing 1408G 16S rRNA are only slightly resistant to paromomycin. There is a moderate decrease (~10-fold) in paromomycin binding affinity for 1408G mutant 30S subunits. It was demonstrated previously that 100 µM paromomycin causes a weak footprint at G1494(N7) in 30S subunits containing the 1408G mutation (Recht et al., 1999). These results agree with those obtained with a model oligonucleotide corresponding to the E.coli decoding region A site (Recht et al., 1996). With an oligonucleotide containing an A1408 to G mutation, paromomycin caused a weak footprint when present at 100 µM (Recht et al., 1996), whereas neomycin, even when present at 1 mM, caused no changes in reactivity at any bases (data not shown). The resistance phenotype is consistent with the footprinting data. A strong footprint is indicative of high-affinity binding of the aminoglycoside, which is presumably a prerequisite for antibiotic action. Resistance therefore arises from decreased aminoglycoside affinity for its ribosomal target.

**Common elements among aminoglycosides**

With the exception of apramycin, the aminoglycosides to which expression of 16S rRNA containing the 1408G mutation conferred resistance all contain a 6’ amino group...
on ring I of the aminoglycoside (Figure 5). The cells are sensitive to aminoglycosides containing a 6’ hydroxyl group (paromomycin and G418). The greatest resistance was observed for aminoglycosides containing the combination of a 6’ amino and a 2’ hydroxyl group on ring I (kanamycin A and amikacin). All of these aminoglycosides contain 2-deoxystreptamine (ring II), which is either 4,5- or 4,6-disubstituted. This pattern of substitution is neither important for binding to the prokaryotic sequence (Yoshizawa et al., 1998) nor for the observed resistance, since 1408G confers resistance to both neomycin and kanamycin (4,5- and 4,6-disubstituted-2-deoxystreptamine, respectively).

**Discussion**

The aminoglycoside-binding site on the 30S subunit is the location of the codon–anticodon interaction on the ribosome. The highly conserved sequences of 16S rRNA that make up the decoding region are essential for ribosome function (Zimmermann et al., 1990). The results presented here demonstrate that functional ribosomes are produced with an A1408G to G mutation in the decoding region A site of E.coli ribosomes. Assuming a conserved mechanism of decoding between prokaryotic and eukaryotic ribosomes, the conservation of nucleotide 1408 within a phylogenetic domain is not critical to ribosome function, as the 1408G mutation can be accommodated by a prokaryotic ribosome. Ribosomes containing a 1408G mutation in 16S rRNA do slow the growth rate of E.coli when they are the only active ribosomes in the cell, whereas no growth phenotype is observed when they are present as the majority of a mixture with wild-type ribosomes. This may be due to reduced activity of the 1408G ribosomes in the presence of the antibiotic spectinomycin. Functional interactions between the 1192 region of 16S rRNA, where spectinomycin binds to the ribosome (Moazed and Noller, 1987), and the decoding region have been observed previously (Hui et al., 1988). Further studies of the kinetics and accuracy of the 1408G ribosomes may help to explain why this sequence is not normally observed in prokaryotic 16S rRNA.

A low level of aminoglycoside resistance was observed even when ~40% of the actively translating ribosomes were sensitive to the antibiotic. This partial dominance of resistance was unexpected, as aminoglycoside sensitivity was dominant to resistance in E.coli heterozygous for resistance to the structurally distinct aminoglycoside streptomycin (Sparling et al., 1968). No resistance to streptomycin was observed for 16S rRNA mutations that confer streptomycin resistance unless the wild-type ribosomes were removed from the actively translating pool by addition of spectinomycin (Powers and Noller, 1991). Aminoglycoside resistance with a mixed ribosome population may be specific to the 2-deoxystreptamine antibiotics, as the binding site of, and rRNA resistance mutations to, these antibiotics are localized to the decoding region A site. The precise location of the binding site for streptomycin is still unclear, and rRNA mutations that confer resistance to it may do so in an indirect manner.

Resistance arises from decreased affinity of a subclass of aminoglycosides for ribosomes with the 1408G mutation. All aminoglycosides to which there is significant resistance have changes in the covalent geometry of ring I. The 1408G mutation perturbs the binding pocket for ring I of the aminoglycosides by changing the conformation of the 1408–A1493 base pair. The 6’ hydrogen bond donor on the aminoglycoside contacts the phosphodiester backbone between A1492 and A1493 in both the gentamicin C1a (6’ NH2) and paromomycin (6’ OH) complex with wild-type rRNA (Fourmy et al., 1996; Yoshizawa et al., 1998) (Figure 6). The 1408G mutation leads to a strong preference for a 6’ hydroxyl in the binding pocket. It is
possible that a 6′ amino group, with its positive charge, altered hydrogen bonding capability and larger van der Waals radius, cannot be accommodated in the altered binding pocket. NMR structural studies on the 1408G mutant RNA indicate a subtle conformational change in the aminoglycoside-binding site (S.R. Lynch and J.D. Puglisi, unpublished), which then leads to large effects on aminoglycoside activity.

Our results are consistent with previous reports of aminoglycoside resistance and in vitro antibiotic activity. Resistance to kanamycin and apramycin in clinical isolates of Mycobacterium species is caused by mutation of A1408 to G (Alangaden et al., 1998; Prammananan et al., 1998). In addition, Streptomycetes tenjimariensis, which produces the aminoglycoside istamycin, methylates the N1 position of A1408 of its 16S rRNA. This methylation precludes the aminoglycoside istamycin, methylates the N1 position on ring I are the most effective in stimulating misreading with eukaryotic ribosomes, whereas aminoglycosides with a 6′ amino group are less active (Wilhelm et al., 1978).

The eukaryotic organisms Tetrahymena thermophila and Giardia lamblia are sensitive to aminoglycosides containing a 6′ hydroxyl group (Palmer and Wilhelm, 1978; Edlind, 1989). The 18S rRNA from these organisms contains a 1409–1419 base pair (E.coli numbering), which is present in prokaryotes but normally absent in higher eukaryotes (Figure 2) (Gutell, 1994). Disruption of this base pair in E.coli has been shown to confer resistance to most 2-deoxystreptamine aminoglycosides, including those containing a 6′ hydroxyl (DeStasio et al., 1989; DeStasio and Dahlberg, 1990). The presence of both 1408G and a mismatch at positions 1409–1491 most likely decreases the affinity of aminoglycosides for the eukaryotic ribosome more than either base substitution alone.

The results presented here indicate that the specificity of aminoglycosides for prokaryotic ribosomes is largely determined by the presence of an adenosine at nucleotide 1408. Since we observe resistance to the aminoglycosides normally inactive against eukaryotic ribosomes by mutation of 1408 to a guanosine in the context of the E.coli ribosome, we conclude that this sequence variation between prokaryotes and eukaryotes is what makes the aminoglycosides useful as antibiotics. These results could aid in the design of new aminoglycosides that possess greater specificity for prokaryotic ribosomes.

Materials and methods

Mutagenesis and expression of plasmid-encoded 16S rRNA

The 1408G mutation was introduced into the plasmid pKK3535, and expressed from the natural rnb promoters as described previously (Recht et al., 1999). This mutation was introduced both with and without the additional mutation 1192U, which confers resistance to spectinomycin (Sigmund et al., 1988), as well as the allele-specific priming site V mutation (Powers and Noller, 1993). All mutant 16S RNAs were expressed from the rnb promoters of pKK3535. Quantitation of plasmid-encoded RNA was performed as described (Recht et al., 1999).

In chemical modification experiments, the priming site V mutation allowed exclusive monitoring of plasmid-encoded 16S rRNA in the mixture of plasmid- and genome-encoded 16S rRNA. The priming site V mutation does not affect the interaction of aminoglycosides with the ribosome (Recht et al., 1999). As a control in all in vivo experiments, the wild-type 16S sequence containing priming site V was expressed from the plasmid pKK3535.

Determination of minimum inhibitory concentration (MIC)

Cultures were started from single colonies and grown in LB media containing spectinomycin for 1192U plasmids or 100 µg/ml ampicillin for 1192C plasmids. MIC tests were performed by inoculation of 5 ml cultures with a 1:1000 dilution of overnight culture in LB media containing spectinomycin (or ampicillin) plus a 2-fold series of dilutions of one of the following aminoglycosides: paromomycin, neomycin (a mixture of neomycin B and C), gentamicin C (a mixture of C1, C1a and C2), kanamycin A, amikacin, apramycin, tobramycin, neamine or G418.

The reported MIC is the concentration of aminoglycoside at which the growth of the cultures was completely inhibited after 24 h incubation at 37°C.

To monitor bacterial growth, cultures were inoculated with a 1:200 dilution of an overnight culture in LB media containing spectinomycin or ampicillin. For experiments in which neomycin was added, cells were allowed to grow until the culture reached an OD600 of ~0.2. Neomycin was added to a final concentration of 20 µg/ml and growth was monitored for an additional 6–8 h.

Chemical modification of 30S subunits

Preparation of 30S subunits and chemical modification experiments were performed as described (Recht et al., 1999). Primer extension from priming site V was performed as described (Recht et al., 1999). Primer extension from the 1513 primer was performed as described (Stem et al., 1988) using a 21 nucleotide DNA primer complementary to nucleotides 1535–1514 of the wild-type E.coli 16S rRNA sequence.

Acknowledgements

We thank Satoko Yoshizawa and Scott Blanchard for helpful discussion, Greg Eason for providing neamine, Stephen Lynch for assistance with figure preparation, and Kam Dahlquist for critical reading of the manuscript. This work was supported by NIH grant GM51266.
References


Received February 23, 1999; revised and accepted April 7, 1999