Evidence That Antibiotics Bind to Human Mitochondrial Ribosomal RNA Has Implications for Aminoglycoside Toxicity*

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Background: Aminoglycosides are toxic to humans.

Results: Aminoglycosides bind to human mitochondrial and bacterial helix69 rRNA with equal affinity, but induce the greatest conformational stability in human mitochondrial helix69.

Conclusion: The binding to human mitochondrial helix69 may contribute to toxicity.

Significance: Understanding aminoglycoside binding to human mitochondrial helix69 is crucial for the development of non-toxic aminoglycosides.

Aminoglycosides are a well known antibiotic family used to treat bacterial infections in humans and animals, but which can be toxic. By binding to the decoding site of helix44 of the small subunit RNA of the bacterial ribosome, the aminoglycoside antibiotics inhibit protein synthesis, cause misreading, or obstruct peptidyl-tRNA translocation. Although aminoglycosides bind helix69 of the bacterial large subunit RNA as well, little is known about their interaction with the homologous human helix69. To probe the role this binding event plays in toxicity, changes to thermal stability, base stacking, and conformation upon aminoglycoside binding to the human cytoplasmic helix69 were compared with those of the human mitochondrial and Escherichia coli helix69. Surprisingly, binding of gentamicin and kanamycin A to the chemically synthesized terminal hairpins of the human cytoplasmic, human mitochondrial, and E. coli helix69 revealed similar dissociation constants (1.3–1.7 and 4.0–5.4 μM, respectively). In addition, aminoglycoside binding enforced conformational stability of the human mitochondrial helix69 by increasing base stacking. Proton one-dimensional and two-dimensional NMR suggested significant and specific conformational changes of human mitochondrial and E. coli helix69 upon aminoglycoside binding, as compared with human cytoplasmic helix69. The conformational changes and similar aminoglycoside binding affinities observed for human mitochondrial helix69 and E. coli helix69, as well as the increase in structural stability shown for the former, suggest that this binding event is important to understanding aminoglycoside toxicity.

Aminoglycoside antibiotics are a class of clinically relevant antibiotics used to treat aerobic Gram-negative and some anaerobic bacilli bacterial infections. Aminoglycosides prevent bacterial protein synthesis by binding to prokaryotic ribosomes with greater affinity than to eukaryotic ribosomes (1, 2). They inhibit ribosomal function through an interaction with an internal loop in helix44 (H44) 2 of the small ribosomal subunit decoding site (3–5). There are several classes of aminoglycosides (6). The 2-deoxystreptamine (2-DOS) containing aminoglycosides include gentamicin, kanamycin, and neomycin classes (ring II in Fig. 1) (6). These aminoglycosides are derived from paromamine (6). The binding of paromamine-derived aminoglycosides to H44 decreases the mRNA translation fidelity and inhibits translocation (4, 7, 8). Other non-paromamine-derived aminoglycosides that contain atypical ring structures include hygromycin, apramycin, and spectinomycin. These aminoglycosides are frequently used in veterinarian medicine (9–11). Although the side chains of these antibiotics can vary widely (6), apramycin and hygromycin contain 2-DOS in common (ring I in Fig. 1, C and D). Both apramycin and hygromycin B have been shown to bind H44 and disrupt translocation of mRNA and tRNA on the ribosome (12, 13). Because of these mechanisms of action, aminoglycosides are effective in treating Gram-negative bacterial infections. However, aminoglycosides are also known to cause ototoxicity and nephrotoxicity in humans (14–17).

It has been established that aminoglycosides bind, in addition to H44, the terminal hairpin of helix69 (H69) of the bacterial large ribosomal subunit (see Fig. 2) (18), which plays a critical role in ribosomal recycling and translocation (19). The H69 nucleotides to which aminoglycosides bind are highly conserved in both sequence and secondary loop structure among bacterial phylogeny (20). The terminal stem-loops of H69 is positioned to be in direct contact with aminoacyl- and peptidyl-tRNAs (A-site and P-site tRNAs respectively) (18, 21) and interacts with the penultimate stem of H44 of the small subunit, forming an intersubunit bridge. In Escherichia coli, ribosomal...
recycling factors (RRFs) aid in the structural rearrangements of the *E. coli* ribosome that enable ribosomal recycling (22). However, aminoglycoside binding suppresses the required conformational changes induced by RRFs and, thus, negates ribosomal recycling (18).

Therapeutic use of aminoglycosides can produce side effects in which mitochondrial dysfunctions are observed in human and animal renal, auditory, and muscular disorders (23–30). However, the biochemical effects of aminoglycosides on human cytoplasmic and human mitochondrial H69 have as yet to be studied. Therefore, we explored and compared the effects of aminoglycoside binding to the human cytoplasmic (hcyt69) and human mitochondrial H69 constructs. The aminoglycosides exhibited similar binding affinities to hmt69, hcyt69, and *E. coli* H69 constructs. Interestingly, aminoglycoside binding effected larger conformational changes of hmt69 residues than hcyt69 and enhanced the conformational stability of hmt69 to a greater extent than that of hcyt69 or *E. coli* H69. The conformational changes and similar aminoglycoside binding affinities observed for hmt69 and *E. coli* H69, as well as the increase in structural stability shown for the former, suggest that this binding event is important to understanding aminoglycoside toxicity.

**Experimental Procedures**

**Sample Preparation**—RNA sequences corresponding to the H69 and H44 species (see Fig. 2) were chemically synthesized (Dharmacon). Because the presence of pseudouridine-modified residues in H69 does not considerably affect aminoglycoside binding affinity, as well as the native structure or stability, the H69 constructs did not include pseudouridine modifications (31–35). The hcyt69 construct corresponds to nucleosides 3722–3740 of human 28S rRNA, whereas the hmt69 construct corresponds to nucleosides 2894–2911 of human mitochondrial 23S rRNA and the *E. coli* H69 construct corresponds to nucleosides 1906–1924 of *E. coli* 23S rRNA. For human mitochondrial H44 (hmt44) and *E. coli* H44 constructs, a hyperstable RNA tetraloop was inserted between the two strands (36–39). The hmt44 construct corresponds to nucleosides 1483–1500 and 1549–1569 of human mitochondrial 12S rRNA connected by a UUCG tetraloop (40). The *E. coli* H44 construct corresponds to nucleosides 1400–1412 and 1488–1502 of *E. coli* 16S rRNA (36) connected by the tetraloop GCAA. All RNA samples were deprotected per the manufacturer’s instructions, extensively dialyzed in H2O (18 megohms), and analyzed for purity and integrity by denaturing polyacrylamide gel electrophoresis. The concentrations were determined by UV absorbance at 260 nm at room temperature. Apramycin (Sigma-Aldrich), gentamicin mixture (C1, C1a, C2, C2a) (AMRESCO), kanamycin A (Fisher Scientific), and hygromycin B (Sigma-Aldrich) were prepared in H2O (18 megohms).

**UV-monitored Thermal Stability Analysis**—UV-monitored thermal denaturation experiments were performed as de-
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Results

Thermal Characterization of H69 Hairpin Constructs—The aminoglycoside binding domain of the E. coli 23S rRNA H69 hairpin (nucleosides 1906–1924) was chemically synthesized, as were the homologous sequences for hcyt69 (nucleosides 19275–19280) and hmt69 (nucleosides 2894–2911). hmt69 and E. coli H69 constructs of native sequence include four canonical G-C base pairs in the same locations, but not orientation (Fig. 2). The hcyt69 construct includes three canonical G-C base pairs without a particular resemblance to the other two constructs. All H69 rRNAs have a wobble pair G-U located at the penultimate position of the stem (Fig. 2). hmt69 has a loop of six nucleosides, whereas the hcyt69 and E. coli H69 hairpins have loops of seven nucleosides each (Fig. 2). The three H69 hairpins behave as unimolecular species when thermally denatured and renatured under moderate ionic strength and pH (20 mM phosphate buffer, pH 6.8) (Fig. 3). The major melting transition from the helix to random coil occurred approximately between 40 and 55 °C for hcyt69, and between 48 and 65 °C for hmt69 and E. coli H69 (Fig. 3). The hmt69 and E. coli H69 demonstrated similar melting temperatures of 61 and 58 °C, respectively, whereas hcyt69 displayed the lowest T_m of 51.6 °C (Table 1). These results are expected considering that the hmt69 and E. coli H69 constructs included a greater number of G-C base pairs than the hcyt69 construct.

Circular Dichroism Spectroscopy—CD spectroscopy experiments were performed in triplicate with a JASCO J815 CD spectrophotometer. Concentrations of gentamicin and kanamycin A up to 40 μM were used to bind H69 (5 μM), hmt64 and E. coli H44 (1 μM) samples were titrated with kanamycin A from 0 to 8 μM. All CD samples were prepared in 20 mM sodium phosphate buffer at pH 6.8. The CD spectra were collected at room temperature from 190 to 320 nm at a rate of 100 nm/min with a resolution of 1 nm and were averaged over six runs in triplicate. The thermodynamic parameters were derived using MeltWin (41). The binding constant (K_d) was calculated by Prism (GraphPad) using the one-site total binding equation,

\[ y = \Delta T_{\text{m(max)}} \cdot \frac{[x]}{(K_d + [x])} + N_S \cdot [x] \]  

where \( y \) is the total binding, \([x]\) is the aminoglycoside concentration, and \( N_S \) is the nonspecific binding proportional to the concentration of aminoglycosides.

NMR Spectroscopy—^H one-dimensional and two-dimensional NMR spectra were collected at 278 K on a Bruker Avance III 500 MHz spectrometer equipped with an ultrasmallest triple resonance cryoprobe capable of applying pulsed field gradients along the z axis. The hmt69, hcyt69, and E. coli H69 samples (300 μM) were dissolved in 10% 2H2O, 20 mM sodium phosphate, pH 6.8. Kanamycin A was directly added to the NMR samples to a final concentration of 1.5 mM. NOESY experiments with water suppression using excitation sculpting with gradients (43) and with 200 and 400 ms mixing times were acquired for assignment of the exchangeable protons. Data were processed using TopSpin 2.1 (Bruker) and NMRPipe (44) and analyzed with TopSpin 2.1 and SPARKY 3 (45).
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The ability of hmt69, hcyt69, and E. coli H69 to bind the paromamine-derived aminoglycosides, gentamicin and kanamycin A, was assessed as well as that of the non-paromamine-derived aminoglycoside, apramycin. Previous studies have shown that binding of aminoglycosides to the bacterial H44 RNA duplex increases the thermal stability of the RNA (46). Therefore, to assess aminoglycoside binding to H69, the thermal stability of the H69 constructs in the absence and in the presence of increasing concentrations of aminoglycoside was analyzed using repeated cycles of UV-monitored thermal denaturation and renaturation (Fig. 4A). The titration of hmt69, hcyt69, and E. coli H69 constructs with kanamycin A and gentamicin exhibited an increase in the $T_m$ of the major thermal transition, indicative of aminoglycoside binding. Each binding curve derived from the change in $T_m$ versus aminoglycoside concentration (Fig. 4, B−D) was fit to one-site nonlinear regression curves. Error bars represent standard error of the mean of three independent UV-thermal stability analysis experiments.

| rRNA       | $T_m$  | $\Delta G$ | $\Delta H$ | $\Delta S$ |
|------------|--------|------------|------------|------------|
| hmt69      | 58.4   | −3.1       | −47.9      | −145       |
| hcyt69     | 51.6   | −2.12      | −47.1      | −145       |
| E. coli H69| 61.3   | −4.47      | −61.5      | −184       |

**TABLE 1**

Thermodynamic parameters of the rRNA helix69 constructs

Each thermodynamic property represents the average of three independent experiments. The coefficient variations of hmt69, hcyt69 and E. coli H69 are less than 5.15%, 5.64%, and 6.53% respectively for all data.

**TABLE 2**

Binding constants of gentamicin to helix69 constructs and changes in thermodynamic properties due to binding

The given values indicate the average of the three independent experiments. The coefficient variations of hmt69, hcyt69, and E. coli H69 titrated with increasing concentrations of gentamicin (1−9 μM) are less than 5.66%, 5.74%, and 5.45%, respectively, for all data. $\Delta G^\circ$, $\Delta T_m$, $\Delta H$, and $\Delta S$ are derived from the difference between $G^\circ$, $T_m$, $H$, and $S$ in the absence of antibiotics and the presence of antibiotic.

| Helix69 rRNA | Gentamicin |
|--------------|------------|
|              | hmt69      | hcyt69     | E. coli H69 |
| $K_d$ (μM)   | 1.68       | 1.69       | 1.31        |
| $\Delta G^\circ$ (kcal/mol, 37 °C) | −4.39    | −3.79      | −5.90       |
| $\Delta T_m$ (kcal/mol, 37 °C) | −1.29    | −1.67      | −1.43       |
| $\Delta T_m$ (°C) | 5.27      | 6.80       | 4.70        |
| $\Delta H$ (kcal/mol) | −7.34    | −11.3      | −7.37       |
| $\Delta S$ (cal/K × mol) | −19.5    | −31.1      | −19.2       |
E. coli H69 constructs were 4.32, 5.35, and 4.02 μM, respectively (Table 3). The 3-fold greater binding affinity of gentamicin is reflective of the more rapid increase in \( \Delta T_m \) during the titration as compared with that of kanamycin A (Fig. 4, B and C). Initially, we hypothesized that \( K_v \) values for E. coli H69 would be lower than the human H69 constructs because aminoglycoside antibiotics preferentially target bacterial ribosomes (2). However, the similar binding affinities to all H69 constructs may be due to aminoglycoside off-target binding and suggest a mechanism for the aminoglycoside toxicity displayed in humans.

The toxic mechanism of aminoglycoside binding to hmt69 may be mediated by threading intercalation of the antibiotics. The binding site of threading intercalation of a stem-binding peptide has been shown to require C-G and G-C base pairs (the two base pairs are in different orientations) directly adjacent to G-U wobble base pair, followed by a loop (47). The C-G and G-C base pairs were proposed to be crucial in providing the site of intercalation (47). hmt69 meets the requirement of the threading intercalation, and, thus, it may be the binding mechanism of the aminoglycoside antibiotics (hmt69 G2895-U2910, C2896–C2898, C2897–C2901, C2902–G2906, G2907–C2908). However, these requirements are unfulfilled in E. coli H69 (E. coli H69 G1907-U1923, C1908–G1922, C1909–G1921). Titration of the H69 constructs with the non-paromamine aminoglycoside, apramycin, caused virtually no change in the melting temperatures of the H69 RNAs (Fig. 4D), indicating that apramycin does not bind to H69. It has been established that apramycin binds in the deep groove of H44, which is composed of noncanonical C-A and G-A base pairs (12). Because H69 constructs do not include such noncanonical base pairs, our results were not unexpected, and further analysis with apramycin was not pursued.

**Structural Effects of Aminoglycoside Binding to the H69 Constructs as Observed by Circular Dichroism—**Surprisingly, investigations of the thermal stability of the aminoglycoside-RNA complexes indicated that gentamicin binds the hmt69, hcyt69, and E. coli H69 constructs with similar \( K_v \) values as kanamycin A. Therefore, we investigated and compared the effects of gentamicin and kanamycin A on the conformation of all H69 constructs using CD spectropolarimetry. Changes in CD ellipticity at 200–220 and 250–290 nm are indicative of conformational changes in the ribose-phosphate backbone and the base region of RNA, respectively (48). In the CD spectra, an increasing ellipticity at base regions of H69 indicated increased base stacking, which is highly correlated to the thermal stability established by aminoglycoside binding. The 2-DOS-containing paromamine-derived aminoglycosides, such as kanamycin A and gentamicin, have been known to form a pseudo-base pair or stack with bases of H44 (49). Also, these aminoglycosides interact with phosphate groups of H44 and stabilize the position of ring I (49). Therefore, the aminoglycoside binding would likely increase the ellipticity of both the phosphate backbone and the base region of H69.

Our CD results illustrated clear distinctions of the aminoglycoside-induced structural effects in the base and the phosphate backbone regions. The three H69 constructs displayed nearly equal increases in ellipticity in the phosphate backbone regions when titrated with increasing concentrations of gentamicin (Fig. 5). However, the greatest significant increase occurred with hmt69. This suggests that although the overall binding affinity of gentamicin to all H69 constructs appears to be similar, gentamicin binding enhances the conformational stability of hmt69 more than hcyt69 or E. coli H69. Upon kanamycin A binding, hmt69 displayed a considerably larger increase in ellipticity for both the base and the phosphate backbone region of the CD spectrum than did hcyt69 and E. coli H69 (Fig. 6). However, the overall ellipticity induced by kanamycin A binding was less than that of gentamicin for all H69 constructs (Figs. 5 and 6). Because kanamycin A binding to hmt69 displayed the largest increase in ellipticity for both the phosphate backbone and the base region, off-target binding to hmt69 might play a more prominent role in kanamycin A toxicity in humans. Together, these data suggest that the binding mechanism of kanamycin A and gentamicin increases the conformational stability of hmt69 and that the resultant conformational stability is greater with hmt69 than that of E. coli H69 and hcyt69.

**Nucleosides Affected by Kanamycin A Binding as Observed by NMR—**Our CD results indicated that kanamycin A and gentamicin binding increase the conformational stability of the hmt69 construct to a greater extent than that of hcyt69 or E. coli H69. Because kanamycin A binding to hmt69 indicated the largest increase in CD ellipticity for both the phosphate backbone and the base region, we further investigated kanamycin A binding to the hmt69, hcyt69, and E. coli H69 constructs using NMR. In this analysis, each RNA construct was titrated with kanamycin A to observe changes in the resonances of base-paired imino protons of the H69 stem. It is known that aminoglycosides bind the stem region, and not the loop of the H69 hairpin (18). These resonances are highly sensitive to changes in environment and dynamics. RNA base-paired imino resonances occupy a region of the NMR
The base-paired imino resonances of the hcyt69, hmt69, and E. coli H69 constructs were assigned using $^1$H one-dimensional and two-dimensional $^2$H-$^1$H NOESY NMR experiments. In the proton spectrum far downfield of other resonances exhibited by RNA or aminoglycosides, allowing them to be monitored unambiguously.

FIGURE 5. Gentamicin exerts differential structural effects on H69 constructs. A-C, CD spectra are the result of 5 μM of E. coli H69 (A), hmt69 (B), and hcyt69 titrated with gentamicin (0–40 μM) (C). The RNA-to-aminoglycoside molar ratios are indicated in panel A (bottom right). The dotted lines are aligned at 274 nm. Each curve represents a point-by-point of six repeats in triplicate. The standard error is less than 5 cm$^2$/mmol for all data between 200 and 320 nm.
FIGURE 6. Kanamycin A produces the greatest structural effects to the hmt69 construct. A–C, CD spectra are the result of 5 μM of E. coli H69 (A), hmt69 (B), and hcyt69 (C) titrated with kanamycin A (0–40 μM). The RNA-to-aminoglycoside molar ratios are indicated in panel A (bottom right). The dotted lines are aligned at 274 nm. Each curve represents a point-by-point average of six repeats in triplicate. The standard deviation is less than 4.7 cm²/mmol for all data between 200 and 320 nm.
one-dimensional $^1$H NMR spectra of hcyt69 and hmt69, seven imino proton resonances were observed between 10.0 and 14.0 ppm for the expected six base pairs of the stems; the *E. coli* H69 construct displayed five resonances in that region (see Fig. 9, A and B, D and E, and G and H). The noncanonical G-U base pair has two imino protons (G3723-U3739, G2895-U2910 and G1907-U1924 for the hcyt69, hmt69, and *E. coli* H69 constructs, respectively; see Fig. 9, A, D, and G) with characteristic upfield shifts, from which the remaining imino proton peaks were sequentially and unambiguously assigned using the cross-peaks present in the same spectral region of the $^1$H-$^1$H NOESY spectrum (see Fig. 9, C, F, and I). To distinguish between the G-U resonances, we noted that in other occurrences of G-U pairs in standard A-form RNA geometry, the distance between the G or U imino protons and the imino proton of the guanosine nucleosides in the adjacent base pair on the loop side (5’ to the G and 3’ to the U) is shorter between the cross-strand than the intrastrand imino protons (50). Correspondingly, the weaker of the two cross-peaks between the guanosine and the G-U imino protons was assigned to the resonance situated on the same strand as the adjacent guanosine. We also note that spin diffusion was observed at long mixing times in certain resonances in the *E. coli* H69 sample and may have affected apparent peak volumes, and therefore G-U assignments, in that sample or others. Neither of the weak resonances at 12.4 and 12.9 ppm for hcyt69, or at 12.4 and 13.3 ppm for hmt69, were observable in the $^1$H-$^1$H NOESY spectra, a common occurrence for fast exchanging imino protons in terminal nucleosides at the ends of stems (50); they are nonspecifically attributed to G3722 and U3727 and to G2911 and U2899, respectively.

Kanamycin A was added to each of the H69 NMR samples to a final concentration of five molar equivalents, as this is the low temperature transition, and thus, reflected the one-site binding (see Fig. 8) observed by others (46). The binding of kanamycin A to the bacterial H44 and hmt44 exhibited $K_d$ values of 2.46 ± 2.89 and 2.99 ± 1.92 μM, respectively. This compares favorably to a binding study using fluorescence anisotropy that demonstrated that the binding affinity of kanamycin B to bacterial H44 was $K_d = 2.14 ± 0.29$ μM (51). With the binding of concentrations greater than 3 μM kanamycin A, the *E. coli* H44 construct no longer exhibited the low temperature transition, and thus, reflected the one-site binding (see Fig. 8) observed by others (46). The $T_m$ of hmt44 also increased due to kanamycin A binding, but the increase in $T_m$ was marginal as compared with *E. coli* H44. These results validate our approach of utilizing UV analysis to understand aminoglycoside binding and further indicate that aminoglycosides may prefer to bind to bacterial H44 than hmt44.

**Structural Effects of Kanamycin A Binding to hmt44 and *E. coli* H44 Observed by CD Analysis**—Given the understanding that the prokaryotic H44 is the primary target of aminoglycosides, we investigated the conformational effects of kanamycin A binding to *E. coli* H44 and hmt44 using CD, as we did for H69. CD spectral results determined that kanamycin A binding increased the ellipticity of *E. coli* H44 more than that of hmt44 in both the phosphate backbone and the base regions of the CD spectra (Fig. 7). Together with the UV-monitored thermal stability measurements (Fig. 8), this suggests that kanamycin A binding produces a conformational stability in the *E. coli* H44. This enhanced structural stability generated by aminoglycoside binding was similarly observed with hmt69. CD spectral analysis with H44 constructs confirmed that one of the salient effects of aminoglycoside binding to *E. coli* H44 is an increase of conformational stability mediated by increased base stacking.

**Discussion**

The main target of aminoglycosides is the prokaryotic H44 rRNA of the small subunit (1, 2). H69 rRNA of the *E. coli* large
subunit was identified as a secondary binding site (18). We hypothesized that the off-target binding to hcyt69 and hmt69 may be important for understanding toxic side effects of paromamine-derived aminoglycosides. UV analysis indicated that aminoglycoside binding affinities to hmt69 and E. coli H69 were similar. NMR analysis also demonstrated that the most significantly perturbed chemical shifts of hmt69 upon the binding occurred in the same respective regions in E. coli H69 and to a comparable degree. CD analysis demonstrated that among H69 constructs, hmt69 exhibited the greatest conformational stability upon the binding. These results support the concept that hmt69 binding of aminoglycoside antibiotics may be important to understand toxicity in humans.

As mitochondria display similarities with bacteria in their protein expression machinery (52, 53), we hypothesized that the mechanism of aminoglycoside binding to the mitochondrial and bacterial ribosomes may be similar. Components of the mitochondrial translational machinery resemble those employed by bacteria, but are distinct from their cytoplasmic counterparts (54). For example, an RRF homolog is present in eukaryotic mitochondria, but absent in the cytoplasm (55–57). Bacterial RRF aids structural arrangements of H69 to enable ribosomal recycling (18); the absence of RRF is lethal (58, 59). In E. coli H69, gentamicin has been shown to bind the H69-RRF complex and suppress structural rearrangement, resulting in ribosomal recycling inhibition (18). The bacterial RRF has a highly conserved region that is known to interact with the H69 loop (nucleosides 1915 and 1916) and the stem (nucleosides 1908–1910) of E. coli H69 (22, 60). NMR results presented here revealed that aminoglycosides interact with the H69 stem nucleosides 2898 and 2897 of hmt69, which correspond to nucleosides 1909 and 1910 of E. coli H69 (Fig. 9). Therefore, the RRF binding site would likely overlap with the aminoglycoside binding site in mitochondria and impair the effects of RRF. This similarity exhibited by hmt69 and E. coli H69 may indicate that the aminoglycoside off-target binding to hmt69 may also
inhibit ribosomal recycling and contribute to toxicity in humans.

The structure and components of the large subunit of human mitochondrial ribosomes, however, also display unique characteristics that differ from those of the bacterial or the cytoplasmic ribosomes (61). Although the protein composition of the large subunit of human mitochondrial ribosomes is substantially increased, the length of human mitochondrial rRNA in the large subunit is half that of the bacterial ribosomes (61). Human mitochondrial tRNA also has reduced or completely absent canonical D- and/or T-loops that form the tRNA elbow (61). In the P-site of the human mitochondrial large subunit, additional elbow-stabilizing elements such as uL5, possibly the homolog of the bacterial protein L5, and helix84 (H84) are deleted (61). However, in the P-site of the bacterial large subunit, the minor groove of the tRNA D stem rests on the minor groove of bacterial H69 (62). Deletions of rRNA domains observed in the human mitochondrial ribosome large subunit may indicate that the large subunit could be more susceptible to aminoglycosides, indirectly affecting misreading and thus causing toxicity; alternatively, fewer binding sites could be protective to mitochondrial translation.

The similarity of binding affinities of aminoglycosides to hmt69 and bacterial H69 with comparable secondary and tertiary structures (Figs. 2 and 10) supports the identification of hmt69 as a likely off-target binding site of aminoglycosides. However, E. coli H69 does not meet the requirement. The crystal structure of E. coli H69 in the presence of gentamicin demonstrated that threading intercalation does not occur when binding to E. coli H69 (18). Because UV analysis indicated similar antibiotic binding affinities for hmt69 and E. coli H69 under equilibrium conditions, an investigation of aminoglycoside on- and off-rates may provide further insights into the possible threading intercalation to hmt69. NMR results showed that one of the foremost chemical shift perturbations in hmt69 by aminoglycoside binding occurs with a guanosine residue, G2897, a residue from the required C-G base pairs for threading intercalation (Table 4). However, NMR analysis also demonstrated that the chemical shift perturbation of residues from the necessary C-G base pairs is not observed in E. coli H69 (Table 4). This suggests that the binding of aminoglycosides to hmt69 may be mediated by threading intercalation, and thus, differ from that of E. coli H69. The binding mechanism to hmt69 may be important to our understanding the toxicity of aminoglycosides.

The majority of adverse effects reported during treatment with gentamicin and kanamycin A, both paromamine-derived aminoglycosides, are similar to the symptoms exhibited in mitochondrial dysfunctions and include renal, auditory, and muscular disorders in both humans and animals (23–29). More acute symptoms are associated with the treatment with gentamicin (27, 30, 63–68). It is possible that aminoglycoside-induced mitochondrial dysfunctions in humans may arise from off-target binding to human mitochondrial RNA, such as hmt69. UV observation of aminoglycoside binding to H69 indicated that gentamicin displays a higher binding affinity.
to H69 constructs than kanamycin A. Based on our CD results, it appears that gentamicin binding to H69 constructs also perturbs the RNA conformation more than kanamycin A. The more severe side effects observed with gentamicin therapy may be associated with the higher binding affinity and nonspecific structural effects observed when binding to H69 of the human mitochondrial and cytoplasmic ribosomes. To further understand the mechanism of aminoglycoside toxicity, it would be interesting to investigate aminoglycoside binding to hmt69 in vivo and observe the resultant toxic effects to mitochondria in cells.

Although hmt69 appears to be the target of clinically relevant paromamine-derived aminoglycosides, our results indicate that apramycin and hygromycin B, non-paromamine aminoglyco-
Aminoglycoside binding with one important exception. The one exception, G2897 of hmt69, also displayed a comparable chemical shift perturbation upon binding, yet the respective residue of bacterial H69 was not affected by aminoglycoside binding. Presently, the role of G2897 of hmt69 in the binding of antibiotic has yet to be revealed.

The data presented here provide a greater understanding about the interaction of aminoglycosides with the E. coli and human cytoplasmic and mitochondrial H69 domains of the ribosome and offer further insight for the development of novel aminoglycoside antibiotics that lack toxicity to humans without compromising antibacterial activity. We propose that off-target aminoglycoside binding to hmt69 results in mitochondrial dysfunctions and contributes to side effects displayed in human patients receiving aminoglycoside antibiotic therapy. The conformational changes and similar binding affinities demonstrated for hmt69 and E. coli H69, in addition to the increased structural stability of hmt69 upon binding, support the hypothesis that aminoglycoside binding to hmt69 is important to our understanding of aminoglycoside toxicity.

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Note Added in Proof—Fig. 5A and Fig. 5B were reversed in the version of this article that was published on June 9, 2015 as a Paper in Press. This error has been corrected. The change does not affect the interpretation of the results or the conclusions.

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