Designer aminoglycosides prevent cochlear hair cell loss and hearing loss

Markus E. Huth,1 Kyu-Hee Han,1 Kayvon Sotoudeh,1 Yi-Ju Hsieh,2 Thomas Effertz,1 Andrew A. Vu,1 Sarah Verhoeven,1 Michael H. Hsieh,2 Robert Greenhouse,1 Alan G. Cheng,1,4 and Anthony J. Ricci1,4

1Department of Otolaryngology — Head and Neck Surgery, 2Department of Urology, 3Department of Pediatrics, and 4Department of Molecular and Cellular Physiology, Stanford University School of Medicine, Stanford, California, USA.

Bacterial infections represent a rapidly growing challenge to human health. Aminoglycosides are widely used broad-spectrum antibiotics, but they inflict permanent hearing loss in up to ~50% of patients by causing selective sensory hair cell loss. Here, we hypothesized that reducing aminoglycoside entry into hair cells via mechanotransducer channels would reduce ototoxicity, and therefore we synthesized 9 aminoglycosides with modifications based on biophysical properties of the hair cell mechanotransducer channel and interactions between aminoglycosides and the bacterial ribosome. Compared with the parent aminoglycoside sisomicin, all 9 derivatives displayed no or reduced ototoxicity, with the lead compound N1MS 17 times less ototoxic and with reduced penetration of hair cell mechanotransducer channels in rat cochlear cultures. Both N1MS and sisomicin suppressed growth of E. coli and K. pneumoniae, with N1MS exhibiting superior activity against extended spectrum β lactamase producers, despite diminished activity against P. aeruginosa and S. aureus. Moreover, systemic sisomicin treatment of mice resulted in 75% to 85% hair cell loss and profound hearing loss, whereas N1MS treatment preserved both hair cells and hearing. Finally, in mice with E. coli–infected bladders, systemic N1MS treatment eliminated bacteria from urinary tract tissues and serially collected urine samples, without compromising auditory and kidney functions. Together, our findings establish N1MS as a nonototoxic aminoglycoside and support targeted modification as a promising approach to generating nonototoxic antibiotics.

Conflict of interest: A patent application on the synthesis of novel aminoglycoside has been filed (application no. 14/212,964; Alan G. Cheng, Robert Greenhouse, Anthony J. Ricci).

Submitted: July 21, 2014; Accepted: November 13, 2014.


In both humans and animals, aminoglycosides cause hearing loss by inducing sensory hair cell degeneration (3, 4). The drug is first transported across the blood-labyrinth barrier to enter the endolymph-filled inner ear compartments (8). It then traverses the mechanotransducer channels or other cation channels to enter hair cells (9–11), resulting in cell demise most likely as a result of the generation of reactive oxygen species (3, 12). Past efforts to neutralize aminoglycoside ototoxicity have emphasized the administration of antioxidants, yet the efficacy of this approach is limited (4, 13–15), in part because of the long half-life of aminoglycosides once they enter hair cells (6).

Alternatively, recent studies suggest that structural differences of the aminoglycoside compounds may contribute to differential ototoxicity (16, 17). In particular, apramycin, an aminoglycoside used in veterinary medicine, is less toxic to hair cells, and its diminished toxicity is correlated with its lower affinity to eukaryotic ribosomes than bacterial ribosomes (16). Although this is a proof of concept that different chemical sites on the aminoglycoside contribute to antimicrobial activity and ototoxicity, several aminoglycoside derivatives exhibit decreased ototoxicity yet lack antibacterial activity (18). Thus, a mechanism to modify aminoglycoside to eliminate its ototoxic effects while preserving its antimicrobial effect remains elusive.

Inner ear hair cells are mechanoreceptors uniquely equipped with mechanotransducer (MET) channels located on their stereociliary bundles (19). Because aminoglycoside transport via the
Results

Novel aminoglycoside derivatives exhibit decreased ototoxicity in vitro. Electrochemical gradients drive cations through the hair cell MET channels (Figure 1A and ref. 24). Although the molecular nature of this channel remains controversial, a significant body of literature exists on the biophysical properties (24, 26–30). Given that aminoglycosides are permeable blockers of the MET channel, we hypothesized that targeted modification to reduce positively charged sites on the aminoglycoside molecule would hinder drug entry into hair cells and thus prevent ototoxicity (9). Using established synthetic methods (31), we first chemically protected 3 of the amine groups on the aminoglycoside sisomicin, a biosynthetic precursor of gentamicin, thus allowing subsequent modification of the N1 and/or N3′′ amine site (Supplemental Figure 1, A and B; supplemental material available online with this article; doi:10.1172/JCI77424DS1). Sisomicin was selected because it is hair cell MET channel is required for drug toxicity (9, 20–23), we have redesigned and modified the aminoglycoside sisomicin based on the biophysical properties of the hair cell MET channels (24) and aminoglycoside-bacterial ribosome interactions (25). Here, we have characterized 9 sisomicin derivatives with diminished ototoxicity in vitro, with 3 of the 9 compounds exhibiting comparable anti–E. coli activities to the parent compound sisomicin. The lead compound, N1MS, showed reduced permeation of the MET channel, likely due to the reduced charge per molecule of N1MS. In a mouse model of aminoglycoside-induced deafness, N1MS was significantly less ototoxic than sisomicin. Finally, N1MS treatment eliminated urinary tract infection in vivo. Collectively, our study characterizes a novel nonototoxic aminoglycoside and also introduces an approach to modifying existing aminoglycosides that can reduce ototoxicity while retaining antimicrobial activity.

Figure 1. Novel aminoglycosides have diminished ototoxicity in vitro. (A) Schematic showing that the nonselective hair cell MET channels are permeable to cations (calcium and aminoglycosides). (B) The sisomicin compound is comprised of 3 rings. Targeted modifications were made to the amine groups on the second (N1) and third rings (N3′′). Three distinct chemical moieties were used to modify sisomicin: MS, PS, and BZ. N1BZ: R1=BZ, R2=H; N1MS: R1=MS, R2=H; N1PS: R1=PS, R2=H; N1,3”BZ: R1=BZ, R2=2BZ; N1,3”MS: R1=MS, R2=2MS; N1,3”PS: R1=PS, R2=2PS; N3”BZ: R1=H, R2=2BZ; N3”MS: R1=H, R2=2MS; N3”PS: R1=H, R2=2PS. (C) Experimental paradigm using cochleae from P4 rats to assess ototoxicity of sisomicin and its derivatives in vitro. AG, aminoglycoside. (D) Representative micrograph of the basal turn of undamaged, cultured cochleae, showing myosin VIIa–expressing outer and inner hair cells (OHC and IHC). (E) Sisomicin-treated (200 μM) cochleae showing dramatic loss of outer hair cells. (F) Hair cells were preserved in cochleae cultured with N1MS (200 μM). (G) Hair cell survival (normalized to undamaged, cultured cochleae) in cochleae (divided into 3 regions [base, middle, and apex]) treated with sisomicin or its derivatives (all 200 μM). Sisomicin caused a basal-apical gradient of hair cell loss, whereas treatment with each of the 9 sisomicin derivatives caused less hair cell loss. (H) Dose-response analyses comparing dosage of sisomicin and N1MS treatment to hair cell loss in the basal turn of the cochlea. Sigmoidal best fits estimated $K_e$ of sisomicin and N1MS to be 100 μM and 1.7 mM, respectively. Data are shown as average ± SEM. $n = 4–10$ for G and H. Scale bar: 25 μm.
Novel aminoglycoside shows diminished entry via hair cell MET channels. Aminoglycosides are permeant blockers of the hair cell MET channels (10, 24, 27). To assess aminoglycoside trafficking across MET channels, electrophysiological measurements of rat cochlear hair cells were performed in vitro. As expected, sisomicin (100 μM) reduced inward currents elicited by mechanical stimulation (Figure 4A). In comparison, current amplitudes in the presence of an identical concentration of N1MS were larger, indicating that N1MS acted as a lower affinity blocker for the MET channels (10, 24, 27). To assess aminoglycoside trafficking across MET channels, electrophysiological measurements of rat cochlear hair cells were performed in vitro. As expected, sisomicin (100 μM) reduced inward currents elicited by mechanical stimulation (Figure 4A). In comparison, current amplitudes in the presence of an identical concentration of N1MS were larger, indicating that N1MS acted as a lower affinity blocker for the MET channels (10, 24, 27). To assess aminoglycoside trafficking across MET channels, electrophysiological measurements of rat cochlear hair cells were performed in vitro. As expected, sisomicin (100 μM) reduced inward currents elicited by mechanical stimulation (Figure 4A). In comparison, current amplitudes in the presence of an identical concentration of N1MS were larger, indicating that N1MS acted as a lower affinity blocker for the MET channels (10, 24, 27).

Furthermore, voltage clamping of hair cells showed decreased inward and outward currents in the presence of sisomicin or N1MS across all voltages tested (Figure 4, A and B). The nonlinear voltage-current relationships after drug treatment (sisomicin or N1MS) suggest that both drugs acted as permeant blockers, whose ability to reduce MET channel currents (and thus block the channel) was diminished at the most negative and positive holding potentials. Dose-response experiments at a holding potential of ~80 mV
KD currents were lower for sisomicin than N1MS (showed that drug concentrations blocking 50% of MET channel (n = 65.0 ± 17.7 and 96.1 ± 9.9 μg/ml; N1MS = 2 and 4 μg/ml; gentamicin = 0.5 and >32 μg/ml; tobramycin = 1 and 4 μg/ml, respectively). (B) Growth of most K. pneumoniae strains, including some ESBL-producing ones (asterisks), were inhibited by N1MS, whereas sisomicin, gentamicin, and tobramycin showed robust activities predominantly against non-ESBL-producing strains. MIC\(_{50}\) and MIC\(_{\geq 100}\): sisomicin = 1 and >32 μg/ml; N1MS = 1 and 128 μg/ml; gentamicin = 0.5 and 64 μg/ml; tobramycin = 0.5 and 32 μg/ml, respectively. (C) Sisomicin, gentamicin, and tobramycin inhibited growth of most P. aeruginosa strains, including drug-resistant strains (asterisks). N9744 is resistant to ciprofloxacin, N9750 is resistant to imipenem, and N9923 and N9749 are multidrug resistant. NIMS was inactive against most P. aeruginosa strains. MIC\(_{50}\) and MIC\(_{\geq 100}\): sisomicin = 0.5 and 8 μg/ml; N1MS = 16 and 64 μg/ml; gentamicin = 2 and 16 μg/ml; tobramycin = 0.5 and 4 μg/ml, respectively. (D) Growth of most S. aureus strains was suppressed by sisomicin, gentamicin, and tobramycin but not N1MS treatment. Asterisks indicate methicillin resistant strains. MIC\(_{50}\) and MIC\(_{\geq 100}\): sisomicin = 0.25 and 0.5 μg/ml; N1MS = 8 and 8 μg/ml; gentamicin = 0.5 and 0.5 μg/ml; tobramycin = 0.5 and 0.5 μg/ml, respectively. Each bar represents one experiment (n = 1).

Figure 3. Sisomicin and its novel derivatives inhibit growth of different bacterial species. (A) Minimal inhibitory concentrations for N1MS, sisomicin, gentamicin, and tobramycin against 10 clinical isolates of E. coli showed that all 4 compounds inhibited growth of most strains. Asterisks indicate ESBL-producing strains. MIC\(_{50}\) and MIC\(_{\geq 100}\): sisomicin = 1 and >32 μg/ml; N1MS = 2 and 4 μg/ml; gentamicin = 0.5 and >32 μg/ml; tobramycin = 1 and 4 μg/ml, respectively. (B) Growth of most K. pneumoniae strains, including some ESBL-producing ones (asterisks), were inhibited by N1MS, whereas sisomicin, gentamicin, and tobramycin showed robust activities predominantly against non-ESBL-producing strains. MIC\(_{50}\) and MIC\(_{\geq 100}\): sisomicin = 1 and >32 μg/ml; N1MS = 1 and 128 μg/ml; gentamicin = 0.5 and 64 μg/ml; tobramycin = 0.5 and 32 μg/ml, respectively. (C) Sisomicin, gentamicin, and tobramycin inhibited growth of most P. aeruginosa strains, including drug-resistant strains (asterisks). N9744 is resistant to ciprofloxacin, N9750 is resistant to imipenem, and N9923 and N9749 are multidrug resistant. NIMS was inactive against most P. aeruginosa strains. MIC\(_{50}\) and MIC\(_{\geq 100}\): sisomicin = 0.5 and 8 μg/ml; N1MS = 16 and 64 μg/ml; gentamicin = 2 and 16 μg/ml; tobramycin = 0.5 and 4 μg/ml, respectively. (D) Growth of most S. aureus strains was suppressed by sisomicin, gentamicin, and tobramycin but not N1MS treatment. Asterisks indicate methicillin resistant strains. MIC\(_{50}\) and MIC\(_{\geq 100}\): sisomicin = 0.25 and 0.5 μg/ml; N1MS = 8 and 8 μg/ml; gentamicin = 0.5 and 0.5 μg/ml; tobramycin = 0.5 and 0.5 μg/ml, respectively. Each bar represents one experiment (n = 1).

 showed that drug concentrations blocking 50% of MET channel currents were lower for sisomicin than N1MS (K\(_{D}\) = 65.0 ± 17.7 and 96.1 ± 9.9 μM, respectively) (Figure 4C). Likewise, at the other negative holding potentials examined, the K\(_{D}\) for sisomicin was significantly lower than that for N1MS (P < 0.0001, 1-way analysis of covariance; Figure 4D). Taken together and considering that N1MS is less positively charged than sisomicin, these data indicate that N1MS is less likely to traverse the hair cell MET channel and thus enter hair cells than the parent compound sisomicin.

**Novel aminoglycosides prevent hair cell loss and hearing loss in vivo.** To compare the ototoxicity of sisomicin and N1MS in vivo, we tested them in a deafness paradigm in mice (37). By challenging 30-day-old normal hearing CBA/CaJ mice with a combination of sisomicin and a loop diuretic (single injection of 175 mg/kg sisomicin followed by 300 mg/kg furosemide; Figure 5A), we induced substantially elevated thresholds in auditory brainstem responses (ABRs) and distortion product otoacoustic emissions (DPOAEs) 1 and 5–6 weeks later (Figure 5, C, E, and F). In contrast, we detected no significant threshold shifts in ABR and DPOAE measurements, except a shift from 8 to 17 dB at the 2 highest frequencies, 1 and 5–6 weeks after N1MS treatment (Figure 5, D–F).

Surface preparation of cochleae from sisomicin-treated mice displayed extensive outer hair cell loss in the middle and basal turns (16.9% ± 10.1% and 11.1% ± 10.5% of saline-treated controls, P < 0.001 for both; n = 9 mice; Figure 6, A and B, and Supplemental Table 3), corresponding to loss of auditory function in those animals. Conversely, cochleae from N1MS-treated animals showed substantially more hair cells than sisomicin-treated animals, and thus less toxicity, 1 week and 5–6 weeks after treatment (Supplemental Table 3), consistent with their auditory measurements, which were comparable to those of saline-injected animals (Figure 5, E and F).

To rule out collateral toxicity by sisomicin and N1MS outside the auditory system, 35 different types of organs were examined 3 days after drug administration. Among 5 sisomicin-treated animals, acute renal tubular epithelial degeneration was detected in 2 animals, whereas no tissue toxicity was detected in any of the 10 N1MS-treated animals (Supplemental Table 4). Moreover, we performed dose-response experiments in vivo to determine drug doses that induced animal death and found that sisomicin was lethal at lower doses than N1MS (LD\(_{50}\); 200 ± 2 mg/kg and 544 ± 7 mg/kg, respectively; Supplemental Figure 4). Based on this, we tested the effects of a higher sublethal dose of N1MS and combined it with loop diuretic challenge (400 mg/kg N1MS and 300 mg/kg furosemide). While sisomicin caused ~100% animal mortality at this dose, >95% N1MS-treated animals survived for over a month under these conditions. After treatment with N1MS at this higher dose, there were marked ABR and DPOAE threshold shifts with cor-
The novel aminoglycoside N1MS effectively supressed bacteriuria (Figure 7, B–D). Moreover, bladder and kidney homogenates collected on day 6 demonstrated that both sisomicin and N1MS reduced bacterial titers in urinary tract issues (Figure 7, E and F). One week after treatment, N1MS- and sisomicin-treated mice exhibited normal ABR and DPOAE waveforms and thresholds and also normal serum creatinine levels (625 μg for both; Supplemental Figure 5). In summary, these results indicate that N1MS is effective at eliminating E. coli–mediated urinary tract infection, without compromising auditory and kidney functions.

Discussion

Aminoglycosides are potent antibiotics that are actively used worldwide, with ototoxicity being the major dose-limiting side effect. Our study uses a novel chemical synthetic approach for alleviating aminoglycoside ototoxicity while preserving anti-
bacterial activity. We modified the aminoglycoside compound based on the notion that hindering aminoglycoside entry via hair cell MET channel prevents ototoxicity (9, 20). The design was based on the biophysical properties of the MET channel (24, 26), whose candidate molecular compositions are still being elucidated (22, 40–42). Nonetheless, electrochemical gradients drive cations through the MET channel; therefore, the conversion of basic amine sites of aminoglycosides into carboxamides or sulfonamides renders them nonbasic (less positively charged) and was predicted to decrease drug entry and subsequent toxicity. Our paradigm produced sisomicin derivatives exhibiting diminished in vitro toxicity relative to the parent compound, with the lead compound N1MS less capable of crossing the MET channels than sisomicin. While the electrophysiological measurements were made in vitro to assess drug entry across the MET channel, the electrochemical gradient present in vivo in the intact cochlea is substantially larger. This difference may in part account for the 17-fold decreased ototoxicity in vitro and 2.3-fold decreased ototoxicity in vivo between N1MS and sisomicin. As the MET channel pore size is estimated at 1.25 ± 0.08 nm at its narrowest segment (24), it is possible that the larger sizes of sisomicin derivatives may have also impeded drug entry. However, the effects of compound dimension are likely limited, as the moieties added were relatively small. Thus, future experiments using bulkier moieties may further delineate the relationships among aminoglycoside dimensions and drug entry and ototoxicity.

A rich body of literature supports the hypothesis that oxidative stress mediates aminoglycoside-induced hair cell degeneration (12, 43), yet the initial events that generate reactive oxygen species are poorly understood. Building upon the knowledge that the human mitochondrial mutation A1555G (GenBank accession no. NC 012920) increases the risk of hearing loss after aminoglycoside treatment (44), recent work has revealed the potential roles of mitochondrial stress in aminoglycoside ototoxicity (16, 45). In particular, work on apramycin, a structurally unique aminoglycoside used in veterinary medicine, suggests that its differential antiribosomal activities against eukaryotic cells versus bacteria contribute to its low ototoxic potential. Moreover, recent work suggests that aminoglycosides induce hair cell toxicity by causing ribotoxic stress and in turn inhibit cytoplasmic protein synthesis (46). While our data support the conclusion that decreased drug entry via MET channels underlies diminished aminoglycoside ototoxicity, we did not determine whether sisomicin and sisomicin derivatives differentially bind eukaryotic and bacterial ribosomes, induce ribotoxic stress, or inhibit protein synthesis in hair cells. One or all of these mechanisms may have also contributed to N1MS’s decreased ototoxicity.

Because aminoglycosides can provide broad coverage of gram-negative organisms, they are widely used when the offending organisms are unknown, such as in the setting of neonatal sepsis or peritonitis (47, 48). Additionally, aminoglycosides are clinically indicated for treating pneumonia caused by P. aeruginosa and urinary tract infection caused by E. coli (2). Differential binding affinities for

---

**Figure 5. Modified aminoglycoside prevents hearing loss.** (A) Schematic of drug treatment to deafen 30-day-old CBA/CaJ mice. (B) Representative ABR tracings in a saline-treated mice showing a 30 dB SPL threshold at 23 kHz. (C) Sisomicin-treated (175 mg/kg) mice showed no detectable responses. (D) In contrast, mice administered with N1MS (175 mg/kg) exhibited normal auditory responses (thresholds = 30 dB SPL). (E and F) ABRs and DPOAEs of animals administered saline 1 week prior (magenta), 175 mg/kg sisomicin 1 week prior (black), 175 mg/kg N1MS 1 week prior (red), 175 mg/kg sisomicin 5–6 weeks prior (green), 175 mg/kg N1MS 5 to 6 weeks prior (blue), and 400 mg/kg N1MS 1 week prior (cyan). Sisomicin-treated animals had elevated ABR and DPOAE thresholds in comparison to saline-treated animals, whereas N1MS-treated animals had robust ABR and DPOAE signals 1 and 5–6 weeks after treatment. High-dose sisomicin treatment was lethal (Supplemental Figure 5). High-dose N1MS treatment caused elevated ABR and DPOAE thresholds. Data are shown as average ± SEM. For E and F, n = 57 for saline control; n = 9–14 for other groups.
bacterial ribosomes may contribute to the selectivity of individual aminoglycosides (49, 50). The findings that modification at the 3′ amine site reduced antibacterial activity and that N1MS is selectively active against E. coli and K. pneumoniae are consistent with such a structure-activity relationship. Although N1MS shows decreased activity against P. aeruginosa and S. aureus, it is conceivable that a higher dose of the less ototoxic N1MS may be used to overcome its diminished antimicrobial activity against these organisms. The effects on nephrotoxicity of such a regimen will need to be elucidated. Alternatively, modifications at the 1 amine site may further separate ototoxicity from antimicrobial activity and restore efficacy against a wide spectrum of bacteria. Thus, future studies should further delineate N1MS’s therapeutic window and the structural activity relationships of additional aminoglycoside derivatives.

Aminoglycoside-induced vestibulotoxicity presents both as a side effect of treatment for systemic infection (4) and as an intended therapeutic effect, such as for Meniere’s disease (51). However, the current study did not directly compare whether sisomicin and its derivatives differ in their vestibular hair cell toxicity; such a comparison may help determine the candidacy of aminoglycoside derivatives as clinical therapeutics.

As ototoxicity limits both the dose and duration of aminoglycoside treatment, our study provides proof-of-concept data supporting targeted modification of aminoglycosides as an effective strategy to prevent iatrogenic hearing loss. Future studies modifying other aminoglycoside compounds may help generate a family of aminoglycoside antibiotics with minimized risk of ototoxicity and thus add to the armamentarium of antibiotics.

**Methods**

**Cochlear cultures.** Cochlear tissues were harvested from P4 rats and cultured as previously described (9). Sterile dissection was performed in Hank’s balanced salt solution or Leibovitz’s L-15 medium (GIBCO, Life Technologies Corp.). Harvested tissues were cultured in Dulbecco’s Modified Eagle Medium/F-12 medium containing 10% heat-inactivated fetal bovine serum (both GIBCO) and 0.01% ampicillin (Sigma-Aldrich) overnight (37°C and 5% CO2) before aminoglycoside treatment. Drug treatment lasted 1 hour, after which organs were incubated in aminoglycoside-free media for an additional 48 hours. Sisomicin sulfate (98% pure, OChem), gentamicin, and tobramycin were used (Sigma-Aldrich). Sisomicin derivatives were synthesized by Nanosyn Inc. (see below).

**Chemical synthesis.** The modified sisomicin derivatives were prepared from sisomicin sulfate, which was first converted into N-6′-p-nitrobenzoylcarbonyl-N-2′,N-3-bis-t-butyloxycarbonyl sisomicin using published procedures (refs. 31, 32, and Supplementary Figure 1A). Because N1 has been demonstrated to react preferentially over N3′ with acid chlorides (31), sulfonation or benzylation occurs selectively at the N1 amine, while both N1 and N3′ are functionalized with the use of excess reagent (Supplemental Figure 1A). For the monofunctionalization of N3′, N-6′-p-nitrobenzoylcarbonyl-N-2′,N-3-bis-t-butyloxycarbonyl sisomicin was further protected as the N-1′-nitrobenzenesulfonyl derivative (Supplemental Figure 1B), followed by sulfonation or benzylation of N3′. Last, deprotection of the functionalized molecules was done using established procedures (31, 52). The final products were purified by preparative high-pressure liquid chromatography (not shown), with their purities determined by mass spectrometry (evaporative light scattering detector) (N1BZ = 100%, N1MS = 99.0%, N1P3BZ = 96.4%, N1,3′BZ = 99.0%, N1,3′MS = 99.5%, N1,3′PS = 98.9%, N3′BZ = 99.3%, N3′MS = 99.0%, and N3′PS = 99.3%). The structural assignments were also confirmed using a Thermo Ultimate 3000 RS UHPLC system.

**Electrophysiology.** P7–9 rat cochlear explants were prepared and whole cell patch recordings were obtained from inner hair cells as previously described (19, 53). The pipette solution contained 125 mM CsCl, 3.5 mM MgCl2, 5 mM ATP, 5 mM creatine phosphate, 10 mM HEPES, 1 mM EGTA, 2 mM ascorbate (pH = 7.2), 280-285 mOsm per kg water, while the extracellular solution contained 140 mM NaCl, 2 mM KCl, 2 mM CaCl2, 2 mM MgCl2, 10 mM HEPES, 6 mM glucose, 2 mM pyruvate, 2 mM ascorbate (pH = 7.4), 300–310 mOsm per kg water. A glass probe affixed to a piezoelectric stack was used to mechanically stimulate the hair bundles; stimulus amplitudes were selected to provide the maximal positive and negative response.

**Immunohistochemistry.** As previously described (9), cultured tissues were fixed with 4% paraformaldehyde in PBS at pH 7.4 for 20 minutes at room temperature. For adult cochlear tissues, fixative was gently infused through an apical bony window and the oval window and then incubated in fixative for 45 minutes at room temperature. EDTA was applied to tissues (500 mM for 30 to 40 minutes), before washing with PBS 3 times. Bony capsules of cochleae were micro-dissected away, and stria vascularis and tectorial membranes were removed to expose the organ of Corti.

Tissues were then immersed in a blocking solution containing 5% bovine serum albumin and Triton-X 1% in PBS for 1 hour. Samples were incubated with anti-myosin VIIa antibody (1:1,000; Proteus Biosci-
Inoculated tubes were incubated at 37°C overnight. The lowest antibiotic concentration (MIC) of the aminoglycoside compounds, we streaked out 50 μl of this suspension (10^7 CFU) was inoculated into 2 ml Müller-Hinton broth, followed by growth at 37°C overnight to reach static phase. Bacteria were then subcultured 1:100 into 5 ml fresh media, followed by growth at 37°C for another 18 hours to reach static phase. Bacteria were then serially diluted 10- to 1,000-fold. Twenty-five μl of each dilution was plated onto MacConkey agar plates. CFUs were counted after growth.

To determine the minimum bactericidal concentration (MBC) of the aminoglycoside compounds, we streaked out 50 μl from aminoglycoside-containing, bacteria-inoculated tubes that exhibited no bacterial growth. Whereas bacterial culture was plated onto Müller-Hinton agar (Becton Dickinson) and incubated them overnight at 37°C. Lack of bacterial growth was used to confirm the MBC for each aminoglycoside compound.

For testing against other bacterial strains, sisomicin and derivatives were sent to the Clinical Microbiology Institute, where MIC's were determined using the above methods.

Mouse model of urinary tract infection. Procedures were performed as previously described (39). Briefly, type 1 pilus expression was induced in the uropathogenic E. coli strain UTI89 by inoculating bacteria into the bladders of female 7- to 8-week-old CBA/CaJ mice by transurethral catheterization. For drug treatment, infected mice were injected subcutaneously with sisomicin or N1MS (25–625 μg/25 g dissolved in 100 μl of PBS) every 2 hours for a total of 5 injections after UTI89 infection.

Aseptically excised bladders and kidneys were minced and homogenized in 400 μl sterile PBS. Freshly collected urine or tissue homogenates were then serially diluted 10- to 1,000-fold. Twenty-five μl of each dilution was plated onto MacConkey agar plates. CFUs were counted after growth.

Microscopic imaging and quantification. Tissues were imaged using a confocal laser scanning microscope (Zeiss LSM 5 Pascal AxioVert 200M, Carl Zeiss AG). The cochlea was divided into 3 regions (base, middle, and apex), and 1 or 2 areas per region were imaged. In every area, outer hair cells were counted.

Bacterial culture. Antimicrobial activity was measured by incubating serial dilutions of sisomicin or derivatives with a constant bacterial inoculum. E. coli XL blue (Agilent Technologies) was incubated in 3 ml Müller-Hinton broth (Becton Dickerson) at 37°C overnight to generate static phase cultures. The following day, incubation tubes were inoculated with 10^8 CFU bacteria. Serial 2-fold dilutions of the aminoglycoside compounds (0.03–64 μg/ml) were added to 2 ml Müller-Hinton broth (54). The inoculated tubes were incubated at 37°C overnight. The lowest antibiotic concentration sufficient to inhibit bacterial growth (an absence of a bacterial pellet at the bottom of the tube) was defined as the minimum inhibitory concentration (MIC). To determine antibiotic efficacy, we used an antibiotic concentration of <4 μg/ml as the criteria, as defined for gentamicin by the Clinical and Laboratory Standards Institute (34).
overnight incubation at 37°C. The limits of detection were 4,000 CFU/ml for urine and 160 CFU per bladder. To determine creatinine levels, 25 μl of serum was collected from each mouse and sent to the Diagnostic Laboratory of Veterinary Service Center at Stanford University.

**Auditory measurements.** ABRs and DPOAEs were measured as previously described with custom hardware based around a data acquisition board (PC0E-6251, National Instruments) driven by software written in MATLAB (Mathworks) (55, 56). Briefly, mice were sedated with a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg). ABR signals were measured from a needle electrode positioned inferior to the tympanic bulla, referenced to an electrode placed at the vertex of the head, and a ground electrode was placed at the hind leg. Tone burst stimuli were delivered with frequencies ranging from 4 to 64 kHz (4, 8, 16, 32, 64 kHz) and sound intensities raised from 10 to 80 dB sound pressure level (SPL) in 10-dB steps. At each frequency and sound level, 260 trials were sampled and averaged.

DPOAEs were measured by a probe tip microphone in the external auditory canal. The sound stimuli were two 1-second sine wave tones of differing frequencies (F2 = 1.22 × F1). F2 was varied from 4 to 46 kHz, and the intensities of 2 tones were from 20 to 80 dB SPL with 10-dB steps. The amplitude of the cubic distortion product was measured at 2 × F1–F2. The threshold at each frequency was calculated to be when the DPOAE was >5 dB SPL and 2 SDs above the noise level. For statistical analyses of both ABR and DPOAE responses, a lack of response is designated 80 dB SPL.

**Histology of organs (necropsy).** All procedures were performed by a board-certified veterinary pathologist at the Diagnostic Laboratory of Veterinary Service Center at Stanford University. First, a complete gross examination of organs and tissues was performed. A comprehensive list of organs and tissues was sampled (Supplemental Table 4), and histologic examination was performed at the histology laboratory. For statistical analyses of both ABR and DPOAE responses, a lack of response is designated 80 dB SPL.

**Statistics.** Data were analyzed using Microsoft Excel (Microsoft), GraphPad Prism, and Origin softwares (OriginLab). Two-tailed Student’s t tests and analysis of covariance were performed. When multiple t tests were performed, Bonferroni correction was included. P < 0.05 was considered statistically significant.

**Study approval.** Sprague Dawley rats (Charles River Laboratories) and CBA/CaJ mice (The Jackson Laboratory) were used. Animal care and all experimental procedures were carried out in accordance with institutional guidelines at Stanford University.

**Acknowledgments**

The authors thank Y. Shi and S. Billings for critical reading; M. Traczewski, S. Brown, L. Alexandrova, S. Lynch, R. Luong, and K. Comstock for excellent technical assistance; and D. Mochly-Rosen, K. Grimes, and other Stanford SPARK members for fruitful discussion. We are grateful for support from NIH/NIDCD (P30DC010363), Swiss National Science Foundation (P30B310635/1 to M.E. Huth), NIH/NIDDK (K08DK087895 to M.H. Hsieh), NIH/NIDCD (K08DC011043 and RO1DC013910), Akiko Yamazaki and Jerry Yang Faculty Scholar Fund (to A.G. Cheng), NIH/NIDCD (R21DC012183 and RO1DC003896), Stanford SPARK program, and Child Health Research Institute funding (to A.J. Ricci).

Address correspondence to: Alan G. Cheng or Anthony J. Ricci, 801 Welch Road, Department of Otolaryngology-HNS, Stanford, California 94305, USA. Phone: 650.725.6500; E-mail: aglcheng@stanford.edu (A.G. Cheng), aricci@ohns.stanford.edu (A.J. Ricci).

Markus E. Huth’s present address is: Department of Otorhinolaryngology, Head and Neck Surgery, University Hospital Bern, Freiburgstrasse, Bern, Switzerland.

Yi-Ju Hsieh and Michael H. Hsieh’s present address is: Biomedical Research Institute, Rockville, Maryland, USA.

---


