Introduction

Bacterial infections commonly overwhelm host defenses and require antimicrobial therapy. For over twenty years and despite newer alternative antibiotics, aminoglycosides remain the mainstay treatment worldwide for numerous disease entities, including pneumonia, peritonitis, sepsis, and genitourinary tract infection (1, 2). The salient features of aminoglycosides include potent antimicrobial activities and minimal drug-related allergies. On the other hand, their major limiting side effects are nephrotoxicity, loss of vestibular function, and permanent hearing impairment, which is detected in approximately 20% of treated patients using conventional audiometry (3, 4). When higher frequency hearing is examined, hearing loss is found in almost half of treated patients (47%) (5). This prevalence may be even higher at longer periods after treatment when considering the prolonged half-life of drug in the inner ear cochlea and the irreversible nature of cochlear hair cell loss (6). Despite these side effects, the World Health Organization recommends aminoglycosides as critically important antimicrobials for clinical use (7). At present, aminoglycoside-induced hearing loss represents one of the most common iatrogenic and preventable forms of hearing loss.

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,1,3 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.

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
Novel aminoglycoside derivatives exhibit decreased 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=MS; N1,3”MS: R1=MS, R2=MS; N1,3”PS: R1=PS, R2=PS; N3”BZ: R1=H, R2=BZ; N3”MS: R1=H, R2=MS; N3”PS: R1=H, R2=PS. (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 $K_s$ 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.

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, to a nonototoxic aminoglycoside and also introduces an approach to modifying existing aminoglycosides that can reduce ototoxicity while retaining antimicrobial activity.
Novel aminoglycosides show 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 than sisomicin. 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

strains (9 of 10), including all 3 extended spectrum β lactamase-producing (ESBL-producing) strains (Figure 3A), which are multidrug resistant and known to be associated with high rates of patient mortality (35, 36). N1MS inhibited 8 of 10 K. pneumoniae strains (including 3 of 5 ESBL-producing), whereas sisomicin was active against 6 of 10 strains (1 of 5 ESBL producers; Figure 3B). Compared with sisomicin, N1MS showed diminished activities against P. aeruginosa and S. aureus species (< 0.05 for both; Figure 3, C and D). While modifying the 3′ amine group abolished antibacterial activities of all 6 sisomicin derivatives (Figure 2), 3 of 6 (N3′PS, N1,3′PS, N1,3′BZ) compounds retained some ototoxic effects (Supplemental Figure 3). Conversely, modifying the 1′ amine alone reduced the spectrum of antibacterial effects; enhanced activity against E. coli and K. pneumoniae, particularly against ESBL producers (Figure 3, A and B); and effectively diminished ototoxicity. Together, our results indicate that chemical modification of sisomicin can dissociate its ototoxicity from antimicrobial activities against E. coli and K. pneumoniae, consistent with previous results on the aminoglycoside apramycin (16). However, the findings that N1MS showed decreased toxicity against hair cells (17 times less ototoxic), P. aeruginosa species (22 times less ototoxic), and S. aureus species (18 times less ototoxic) suggest that such dissociation may be species specific. Whether loss of antibacterial activity results from decreased entry into bacteria (2) and/or diminished interaction with bacterial ribosomes remains to be determined.

Novel aminoglycoside derivatives show antibacterial activities in vitro. Enterobacteriaceae rank among the most frequently isolated organisms from patients (33). Based on this, we next tested the antimicrobial activities of sisomicin and its derivatives against E. coli in vitro. Sisomicin and 2 sisomicin derivatives (N1MS and N1PS) showed comparable minimal inhibitory and bactericidal concentrations (using a concentration of <4 μg/ml to define antimicrobial activity; ref. 34 and Figure 2). All 6 compounds modified at the 3′ amine site showed diminished antimicrobial effects (N3′MS, N3′PS, N3′BZ, N1,3′MS, N1,3′PS, N1,3′BZ; Figure 2).

Because N1MS displayed the most favorable antibacterial activity-ototoxicity profile, it was further tested against clinical isolates of E. coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, and Staphylococcus aureus. Similar to sisomicin, gentamicin, and tobramycin, N1MS effectively suppressed growth of most E. coli

commercially available at high purity (>98%) as compared with gentamicin. We selected 3 moieties (methylsulfonyl [MS], phenyl-sulfonyl [PS], and benzoyl [BZ]) to modify these 2 amine groups (1 and 3′) (Figure 1B and Supplemental Figure 1B), which were predicted by X-ray crystallography as dispensable sites for the bacterial ribosome binding-dependent antimicrobial effects of aminoglycosides (25). These functional groups were conjugated to the 1 amine group (N1MS, N1PS, N1BZ), 3′ amine group (N3′MS, N3′PS, N3′BZ), or both the 1 and 3′ amine groups (N1,3′MS, N1,3′PS, N1,3′BZ) of sisomicin (Figure 1B, Supplemental Figure 1C, and Supplemental Figure 2). The chemical synthetic schemes are detailed in Supplemental Figure 1 and ref. 32).

Like gentamicin, sisomicin (200 μM for 1 hour) caused a basal-apical gradient of hair cell loss in in rat cochlear cultures (Figure 1, C–H, and Supplemental Table 1). At the molar equivalent of the parent compound sisomicin (200 μM), all 9 new sisomicin derivatives induced markedly less hair cell loss, with 4 compounds showing almost no hair cell toxicity (Figure 1G and Supplemental Table 1). Conjugation with the MS moiety was more effective than that with either the PS or BZ structures in ameliorating ototoxicity (Supplemental Figure 3, A–C), and all 3 modifications of the sisomicin backbone with the MS moiety eliminated ototoxicity (Supplemental Figure 3, D–F). Dose-response experiments (drug exposure for 1 hour, as above) showed that N1MS (>95% pure, Supplemental Figure 2) is 17 times less toxic than sisomicin (98% KD = 100 ± 2 μM and 1.7 ± 0.2 mM, respectively; Figure 1H and Supplemental Table 2). These data suggest that specific modification of aminoglycosides effectively reduced toxicity against hair cells in vitro.

Novel aminoglycoside derivatives show antibacterial activities. The MICs and MBCs of sisomicin and 9 derivatives were quantified. Using 4 μg/ml (drawn as dotted line) as the criteria set forth by the Clinical and Laboratory Standards Institute, we found that sisomicin, N1MS, and N1PS robustly inhibited growth of and killed E. coli. Data are shown as average ± SEM, n = 5.
showed that drug concentrations blocking 50% of MET channel currents were lower for sisomicin than N1MS (Figure 4C). Likewise, at the other negative holding potentials examined, the \( K_d \) for sisomicin was significantly lower than that for N1MS (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 3). 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-
Corresponding hair cell degeneration (Figure 5, E and F, and Supplemental Table 3). These in vivo data indicate that the sisomicin derivative N1MS is substantially less toxic than sisomicin, corroborating the in vitro findings that modification of aminoglycosides markedly reduced their ototoxicity.

**Novel aminoglycosides eradicate urinary tract infection.** *E. coli* is the most common pathogen causing urinary tract infections (38). To test whether N1MS is effective against urinary tract infections, we inoculated mouse urinary tracts with a uropathogenic strain of *E. coli* (39) followed by treatment with 5 sequential injections of sisomicin or N1MS (Figure 7A). In comparison with saline-injected controls, from which serially collected urine consistently yielded high titers of *E. coli*, both sisomicin and N1MS effectively eliminated bacteria from urine collected 1, 4, and 6 days after inoculation (Figure 7, B–D). All 3 concentrations of N1MS (25, 125, and 625 μg at 5 doses per 20- to 25-g animal) effectively suppressed 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-

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**Figure 4.** Hair cell MET responses at different membrane holding potentials after sisomicin and N1MS treatment. (A and B) Mechanical stimulation (a negative step of 650 nm for 0.1 ms, then a positive step of 650 nm for 5 ms) was used to stimulate hair cell hair bundles at different membrane holding potentials (stepped from -180 to +120 in 20-mV increments). Responses to mechanical stimuli were obtained at different holding potentials to generate the current responses shown in the absence and presence of sisomicin (red) or N1MS (blue, both at 100 μM). Sisomicin reduced current amplitudes more effectively than N1MS and thus acts as a higher affinity blocker for the MET channel. Current-voltage plots show that sisomicin reduced both inward (at negative potentials) and outward currents (at positive potentials) and displayed loss of block at both extreme negative and positive steps, indicating that sisomicin acts as a permeant blocker of the MET channel. Measurements under identical conditions found that blockage with N1MS was markedly diminished. (C) Remaining fraction of control current for sisomicin and N1MS at a holding potential of -120 mV at different concentrations (10, 30, 100, 300, and 1,000 μM). (D) *K*<sub>d</sub> derived from remaining control current fraction for sisomicin and N1MS at different holding potentials and concentrations (-180 to +120, 20-mV increments). Data are shown as average ± SD. *n* = 2–9.
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 sisomicin, 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...
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 Menière’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 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-butylxycarbonyl sisomicin using published procedures (refs. 31, 32, and Supplemental Figure 1A). Because N1 has been demonstrated to react preferentially over N3′ with acid chlorides (31), sulfonylation or benzoylation 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-butylxycarbonyl sisomicin was further protected as the N-1′-p-nitrobenzenesulfonyl derivative (Supplemental Figure 1B), followed by sulfonylation or benzoylation 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%, N1PS = 96.4%, N1,3′BZ = 99.0%, N1,3′MS = 99.5%, N1,3′P5 = 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-
eneces) in blocking solution overnight at 4°C. The following day, tissues were rinsed with PBS and incubated with a fluorescent secondary antibody (Alexa Fluor 555 goat anti-rabbit; Invitrogen, Life Technologies Corp.) in blocking solution for 1 hour. A subset of tissues was also immersed in phalloidin (Alexa Fluor Phalloidin, 1:1,000; Invitrogen) and coverslipped for further imaging and analysis.

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).

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 during MIC assays onto Müller-Hinton agar (Becton Dickerson) 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 pili expression was induced in the uropathogenic *E. coli* strain UT89 by inoculating bacteria into 5 ml Luria-Bertani 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 again. These cultures were centrifuged for 10 minutes at 13,000 g, resuspended in 2 ml PBS, and then diluted to approximately 2 × 10^8 CFU per ml. Fifty μl of this suspension (10^8 CFU) was inoculated 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) dissolved in 100 μl of PBS) every 2 hours for a total of 5 injections after UT89 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...
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 (PC00e-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 then organs and tissues were fixed in 10% neutral buffered formalin. After 48 to 72 hours of formalin fixation, the organs and tissues were trimmed and processed routinely for light microscopic examination. Specifically, 4-micron thick, hematoxylin and eosin–stained tissue sections were evaluated using an Olympus BX-41 microscope (Olympus).

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


Research Article


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