Supplemental items:

Supplemental Methods:

Separation of gentamicin lot containing gentamicin C1, C1a, C2, and C2a (Vendor E, Chem Impex Lot #20110415).

Salt to free base: 1 g of gentamicin sulfate was dissolved in 20 mL of deionized H_2O. Dowex 550A OH anion exchange resin was added to the clear solution until a ~pH11 was reached (pH paper). The resin was filtered and washed with water, and the aqueous filtrate was concentrated to a foamy white solid.

Solvent preparation: 500 mL of isopropyl alcohol (IPA), 500 mL of 17% NH_4OH, and 1L of $CHCl_3$ were sequentially added to a separatory funnel, shaken, and allowed to settle until both layers were clear. The bottom layer was collected and used as the eluent. The upper aqueous layer was discarded.

Chromatographic separation – C1 pure, C2/2a mixture, C1a pure: The free based gentamicin mixture was dissolved in methanol and slurried with an equal weight of flash silica gel, and then the solvent was evaporated to dryness and thoroughly dried under vacuum. The intimate mixture was loaded into a precolumn which was attached to a prepacked column (12 g) of flash silica gel. The column was then eluted with 1:2:1 IPA:$CHCl_3$:17% NH_4OH, at 40 mL/min over 40 min utilizing a Teledyne ISCO combiflash companion system. In total 96 fractions were collected and visualized by iodine stain on TLC. After visualization it was found that C1 and C2/C2a had eluted. The column was flushed with 1:2:1 IPA:$CHCl_3$:28% NH_4OH in order to elute the remaining C1a component of the mixture. The appropriate fractions were collected and reduced under vacuum. Fractions 76-83 contained 107 mg of Gentamicin C1, fractions 86-92 contained 80 mg of C2 and C2a and fractions 6-10 after the solvent swap were combined to give 132 mg of gentamicin C1a. The mixture of gentamicin 2 and 2a were separated by preparative HPLC as illustrated below.

Preparation of sulfate salts: Free based purified gentamicin C-subtypes was dissolved in methanol, and 1M H_2SO_4 was added dropwise until the pH of the solution reached 3. The solution was allowed to stand for 10 min, and the white precipitate was collected via filtration and washed with methanol. The solid was dried under vacuum.

Separation of micronomicin lot containing gentamicin C1a and C2b (Vendor B, Alfa Aesar, Lot #N17C046)

Salt to free base: 5 g of micronomicin sulfate was dissolved in 100 mL of DI H_2O, and 63.96 g of Dowex 550A OH anion exchange resin was added to the clear solution until a pH of ~11 was reached (pH paper). The resin was filtered off and washed with water, and the aqueous filtrate was concentrated to afford 3.03 g of a foamy white solid.

Solvent preparation: 1 L of IPA, 1 L of 17% NH_4OH, and 2 L of $CHCl_3$ were sequentially added to a separatory funnel, shaken, and allowed to settle until both layers were clear. The bottom layer was collected and used as the eluent. The upper aqueous layer was discarded. In the case of the 1:2:1 IPA $CHCl_3$: 28% NH_4OH eluent, the same procedure as above was followed, but using 28% NH_4OH in place of 17% NH_4OH.

Chromatographic separation – C2b and C1a pure: The free based micronomicin mixture was dissolved in methanol and slurried with an equal weight of flash silica gel, and then the solvent was evaporated to dryness and thoroughly dried under vacuum. The intimate mixture was loaded into a precolumn which was then attached to a prepacked column (40 g) of flash silica gel. The mixture was then eluted with 1:2:1, IPA:$CHCl_3$: 17% NH_4OH, at 40 mL/min utilizing a Teledyne ISCO combiflash companion system until TLC analysis indicated that the gentamicin C2b had completely eluted. In total, 90 x 20 mL fractions were collected and visualized by iodine stain on TLC. The column was then flushed with 1:2:1, IPA:$CHCl_3$: 28% NH_4OH in order to elute the remaining C1a component of the mixture. In total 120 x 20 mL fractions were collected.

Similar fractions were combined and concentrated under vacuum to provide 1.98 g of C2b and 0.68 g of C1a as white foams.

Large scale separation of micronomicin – gentamicin C1a and C2b.

Salt to Free Base: Micronomicin sulfate (25.22 g) was dissolved in 500 mL of water and Dowex 550A resin (325 g) was added and stirred overnight until a pH of ~11 was reached. The resin was filtered, and the volatiles removed to give 17.81 g of a white foam.

Chromatographic separation – C2b and C1a pure: The mixture was dry packed into a silica gel precolumn as described above and a similar procedure to the small scale was followed using a prepacked flash column (330 g) and a flow rate of 100 mL/min. In this way, 10.6 g of the gentamicin C2b free base and 2.5g of the gentamicin C1a free base were recovered.

Sample procedure for the preparation of the sulfate salt: The pure free base gentamicin C-subtypes was dissolved in methanol, and 1M H_2SO_4 was added dropwise until the pH of the solution reached 3. The solution was allowed to stand for 10 min, and the white precipitate was collected via filtration and washed with methanol. The solid was thoroughly dried under vacuum. Thus 1.98 g of gentamicin C2b free base afforded 2.10 g of C2b sulfate. gentamicin C1a (0.68 g) provided 1.04 g of C1a sulfate as a white powder.

Isolation of gentamicin C2 and C2a from the mixture by preparative HPLC: The mixture of gentamicin C2 and C2a obtained above from the Chem Impex Lot above was separated by multiple injections of 200 mg each (45 injections, 9 grams of mixture in total). The conditions used were as follows: Column: X-Bridge Prep C18, 10µm OBD 50x250mm Buffer A and B: 97% dH_2O + 3% MeOH +0.2% TFA Gradient: 50% B Isocratic, 60 min, 60 mL/minute flow rate, Loading: 200 mg dissolved in 3.0 mL dH_2O**.** Using this method, gentamicin C2 (RT = 11.42 min) and C2a (RT = 14.83 min) were isolated in pure form and converted to the sulfate salt as described in previous examples. The salts were thoroughly dried under vacuum.

Impurities The impurities in commercial gentamicin, sisomicin, geneticin, gentamicin A, gentamicin X and gentamicin B, were purchased from commercial sources (Supplemental Table 1). Compounds were further validated and characterized using ^{1}H and ^{13}C NMR spectroscopy performed at the Stanford Core Facility (Supplemental Figure 1 and Table 2). One lot of clinical grade gentamicin was obtained from the Stanford Hospital Pharmacy and prepared for this study by evaporation of the injectable aqueous solution to a dry powder form using a desiccator and stored for an additional 48 hrs in a desiccator charged with silica gel.

NMR: ^{1}H and ^{13}C NMR was performed at Stanford University NMR Core Facility. ^{1}H NMR data were collected on a Varian Unity Inova 600 MHz spectrometer, operating at 599.78 MHz, equipped with a 5mm Z-axis PFG Triple Resonance HCN Probe, running VNMRJ 4.2 software. ^{13}C NMR data were collected on a Varian Unity Inova 500 MHz spectrometer, operating at 499.75 MHz for 1H, 125.67 MHz for ^{13}C, equipped with a 5mm Z-axis PFG Switchable X Probe, running VNMRJ 4.2 software. NMR samples were prepared with ~10mg of material in 500 ml of D_2O. All NMR data were collected at 25°C.

^{1}H NMR data were acquired with 16 scans of a 45° pulse of 3.1 ms for an acquisition time of 4 seconds and a recycle delay of 1 second. The data were acquired over a spectral width of 9597 Hz (16 ppm) of 76776 complex points with the transmitter offset set at 5.00 ppm. Fourier transformed data were manually phased and a drift correction was applied to flatten the baseline. Spectra were referenced to hydrogen on demand (HOD) resonance at 4.77 ppm at 25°C.

^{13}C NMR data were acquired with ~200 scans of a 45° pulse of 6.5 ms for an acquisition time of 1.5 seconds and a recycle delay of 1 second with WALTZ-16 1H decoupling. The data were acquired over a spectral width of 33003 Hz (263 ppm) of 99010 complex points with the transmitter offset set at 95.6 ppm. Data were zero-filled to 128K points and processed with line-broadening apodization of 2 Hz. Fourier transformed data were manually phased and a drift

correction was applied to flatten the baseline. Spectra were referenced indirectly to 0 ppm for tetramethylsilane (TMS) in $CDCl_3$. The ^{13}C peak assignments shown in Figure S1 were made using previously published datasets (1, 2). Data analysis was performed in VNMR J4.2 software (3).

Image Acquisition and analysis:

Z-stack images for cell counting were captured using a 40x oil objective (1.3NA) on a Zeiss LSM880 or using a 40X oil objective (1.3NA) on a Zeiss LSM700 confocal microscope. Parameters: pinhole 1 airy unit, resolution 512 x 300 (2121.55 x 124.54μm; ~2.5 pixels per μm), 1 μm step size stacks (<40 slices), data from two imaging channels were taken simultaneously. Imaging was automated using a Zeiss macro software tool and the Zen black 2.3 software. To select the region of interest, an image of the entire slide was first acquired using a 20X dry objective (0.5NA) using tile scanning and DAPI fluorescence only, and a mid-apical imaging region was selected for each sample. Hair cells were counted manually and blindly using FIJI opensource software (NIH https://imagej.nih.gov/) (4). Outer hair cells were identified using the following criteria: presence of a bundle, presence of F-actin and Myosin VIIa at the cuticular plate, non-pyknotic nucleus, and Myosin VIIa expression throughout the cell body and surrounding the nucleus. For inner hair cells, Myosin VIIa and nuclear staining were the criteria since inner hair cells were only used as a metric of distance. Distance was measured along the tunnel of corti using FIJI; distance was used when the inner hair cells were lost or too damaged to count (4). Data points presented as mean ± SD. For each dataset, each drug was normalized to the hair cell counts for the 30 μM dose (undamaged). All but one data set (G418) was fitted with the following Hill1 function using OriginPro2018 software:

$$y = 1 + (0 - 1)\frac{x^n}{k^n + x^n}$$

Geneticin was fitted with the following bi-phasic sigmoidal dose response function:

$$y = A1 + (A2 - A1)\left(\frac{p}{1+10^{(LOGx01-x)h1}} + \frac{1-p}{1+10^{(LOGx02-x)h2}}\right)$$

From the Hill function-fitted data, the EC50 and Hill co-efficient values were extracted. Logx01 is the first EC50, Logx02 is the second EC50, H1 is the first slope, H2 is the second slope, p is the proportion in each dose response and was 0.5, and A1 and A2 are the start and end set as 1 and 0, respectively. Comparisons between fitted data-sets were made using F-Tests.

Antimicrobial Testing:

All tests were performed by the Clinical Microbiology Institute laboratory. Ten bacterial strains were tested from four species - *E. coli*, *S. aureus*, *K. pneumoniae*, and *P. aeruginosa*. For the 40 strains tested, six strains were from the American Type Culture Collection (ATCC, Manassas, VA) and the remaining 32 strains were clinical isolates available from the Clinical Microbiology Institute's collection (Wilsonville, OR). Of the 40 strains, 15 strains were known to have resistance to other antibiotics (e.g., methicillin resistant, ESBL (extended-spectrum beta-lactamases) positive, AmpC beta-lactamase positive, multi-drug resistant, ciprofloxacin resistant and imipenem resistant), and 25 strains were wild-type isolates with no reported antibiotic resistance phenotypes. The minimum inhibitory concentration (MIC) was established for each drug using a microdilution test performed according to the latest Clinical Laboratory Standards Institute (CSLI) document M7-A11, 2018 (31). Briefly, 96 well plates were prepared 1 inoculum for each organism tested, the drug and the medium then added. Inhibition readings were performed after a 24hr growth period (31).

Drugs were prepared as follows, 15 mg of each individual gentamicin component was prepared in a 1 ml Eppendorf tube, and re-suspended in 1ml MilliQ H_2O. Samples were then desiccated for 24 hrs in a Thermo Savant 120 SpeedVac Concentrator (ThermoScientific) and for 48 hrs in chamber containing Drierite (Sigma-Aldrich). The sample was then transferred to a

15 ml falcon tube, the dry weight recorded. For transport, falcon tubes were sealed with parafilm and transported at room temperature (RT).

For data analysis, the CLSI and Hospital Gentamicin breakpoints for *E. coli, S. aureus, K. pneumoniae* and *P. aeruginosa* were used (5). The breakpoint of ≤4 µg/ml for gentamicin is set by CLSI (6). For analysis of potency, geometric means were used to graphically represent the central tendency of the logarithmic categorical data shown in box plot form. For the comparisons between the hospital gentamicin and the individual C-subtypes and impurities, only the strains that were inhibited by hospital gentamicin were compared. Statistical comparisons were performed using a non-parametric Mann-Whitney U-test using OriginPro2018 software (OriginLab, Northampton, MA).

Immunohistochemistry:

Tissues were fixed in 4% paraformaldehyde (PFA) prepared in a 1X phosphate buffered saline (PBS) solution (Electron Microscopy Services, Cat#19342-10) for 30 min at RT. Tissues were then washed with PBS 3 times for 5 min each, then blocked with 5% donkey serum, 0.1% Triton X-100, 1% bovine serum albumin (BSA), (Thermo Fisher Scientific, Cat#9048-46-8), and 0.02% NaN_3 (Sigma-Aldrich) in PBS at pH 7.4 for 1hr at RT. Myosin VIIa polyclonal antibody (1:500, Proteus Biosciences, Cat#25-6790) in the above blocking solution was added overnight at 4°C (57, 58). The following day, the primary antibody was removed using 3 PBS washes. Tissues were then incubated with 1:400 546nm donkey anti-mouse secondary antibody (Life Technologies, #A10036) and counterstained with 1:600 Alexa Fluor 488 Phalloidin (Life Technologies, Cat#A12379) and 1:10,000 of a 5mg/ml 4',6-Diamidino-2-Phenylindole, Dihydrochloride stock solution (Thermo Fisher Scientific, Cat #D1306) diluted in 0.1% Triton X-100, 0.1% BSA, and 0.02% NaN_3 solution in PBS at pH 7.4 for 90 min at RT. Immunohistochemistry was performed while the tissue was secured to the minutien pin.

For imaging, the organ of Corti was dissected away from the modiolus as one unit and explanted onto Cell-Tak (Fisher Scientific, Cat#CB40240) coated, 22 mm diameter round glass coverslips (Bioscience Tools, Cat# CS-No1-10), oriented so that the apical surfaces of the hair cells were pointing upwards. Two organ of Corti explants were placed on each coverslip; 10 coverslips per slide. Approximately 15 µl of prolong diamond antifade mounting media (Life Technologies, Cat# P36970) was added to each coverslip and an additional coverslip added on top. Samples were stored light protected at RT for 48hrs to allow the medium to dry.

Cochlear Cultures:

Post-natal day five (P5) Sprague Dawley rats (weight range: 13.0-16.5 g; mixed genders; Cat#Crl:SD) from Charles River. Housing conditions and experimental procedures used in this study were in accordance with the Guidelines for the Care and Use of Laboratory Animals of the National Institutes of Health and in accordance with Stanford University Administrative Panel on Laboratory Animal Care (APLAC). Rats were decapitated and the head was then surface sterilized by immersion in 70% ethanol for three 30s periods (7, 8). The head was air dried for 30 seconds, then bisected along the mid-sagittal plane and placed into standard HBSS (Cat #14025076, Thermo Fisher Scientific) containing 1.26mM calcium chloride and 0.5mM magnesium chloride for microdissection. The otic capsule was dissected out from the temporal bone and placed in DMEM/F12 (pH-7) containing L-glutamine and 15 mM HEPES (Cat#D8062, Sigma-Aldrich). The cartilaginous capsule surrounding the cochlea was removed and a stainless-steel minutien pin (0.02 mm tip diameter, 0.2 mm rod diameter, 10 mm length; Cat#26002-20, Fine Science Tools) was placed through the modiolus. The stria vascularis was removed from the apex to the base. The organ of Corti, greater epithelial ridge and spiral ganglion remained as one complete piece. The minutien pin was secured to a custom-made sterile plastic tissue chamber (5-6 mm depth and 9 mm diameter; 200 µl volume and was transferred to a 24-well plate containing 1 ml of DMEM/F12 supplemented with 10 µM sodium

pyruvate (Cat#11360-070, Thermo Fisher Scientific) and 20 μM penicillin G (Cat# P3032, Sigma-Aldrich).

Solutions containing aminoglycosides were stored at -80°C, thawed and desiccated for 48 hrs in a container with Drierite (Sigma-Aldrich) prior to each experiment (to remove water content). Aminoglycoside powders were re-suspended in a DMEM/F12 solution supplemented with 100 μM sodium pyruvate and 30 μM Penicillin G. Concentrations ranging from 5 mM to 30 μM were prepared by serial dilution. No growth factors were used during the drug treatment period. For Mix 1 and Mix 2, 100 mg/ml solutions were made in Milli-Q water for each individual component. Mixtures containing the following ratios of components were made (Mix 1: C1a = 0.24, C1 = 0.53, C2 = 0.15, C2a = 0.06, C2b = 0.02; Mix 2: C1a = 0.28, C1 = 0, C2 = 0, C2a = 0.07, C2b = 0.65). Mixtures were then desiccated for 24 hrs in a Speedvac concentrator (ThermoScientific) and 48 hrs in a chamber containing Drierite (Sigma-Aldrich).

For treatments, drugs were applied by removing and adding treatment volume above the height of the tissue chamber; three 1 ml volumes were sequentially removed and replaced for a single treatment. Treatments were gently added using a 1 ml syringe and 18G x 1 ½" needle. Tissues were incubated with drug solution at 37°C, at 5% CO_2 for 1 hr (Thermo Scientific Steri Cycle 370 CO_2 incubator), after which three 1 ml volumes of DMEM/F12 were sequentially removed and replaced to remove each single drug treatment. A final 1 ml of DMEM/F12 solution supplemented with 100 μM sodium pyruvate, 30 μM Penicillin G, 20 ng/ml human epidermal growth factor (Sigma-Aldrich Cat# E9644), 10 ng/ml fibroblast growth factor basic (Sigma-Aldrich, Cat#4777), 55 ng/ml insulin-like growth factor (Sigma-Aldrich, Cat#I8779), and 55 ng/ml heparin sulfate proteoglycan (Sigma-Aldrich, Cat#4777), 1x B-27 supplement (Gibco, Cat#17504-044) and 1x N-2 (Gibco, Cat#17502-048) was then added. Tissues were incubated at 37°C and 5% CO_2 for 46-48 hrs. Cochleae from the same animal were given different drug treatments, cochleae were otherwise randomly distributed.

Electrophysiology: Outer hair cells were recorded from postnatal day 7-10 (P7-P10) rats as previously described (9). Briefly, rats were decapitated and the cochlea was removed and placed into external solution containing (in mM): 142 NaCl, 2 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 6 Glucose, 2 Ascorbate, 2 Pyruvate, 2 Creatine monohydrate, balanced to pH 7.4 with NaOH and a final osmolality of 304 - 310 mOsm. Organ of Corti were further dissected out and a mid-apical region placed into a glass-bottomed recording chamber, held in place with single strands of dental floss. The recording chamber was placed onto the stage for a BX51 fixed stage upright microscope (Olympus) and viewed with a 60x water immersion lens (NA 1.0). The bath was perfused at 2-4 ml/min with a peristaltic pump (Gilson). An apical perfusion pipette was used for drug delivery. The 50-100 µm tipped pipette was placed at a 45-degree angle toward the open face of the sensory hair bundles. Drug selection was through a custom designed and built solenoid switching devices that allowed selection between 7 solutions (37). Solution exchange took less than 2 minutes. Drugs were added to the external solution and serially diluted to desired concentrations. OHCs were accessed by first creating a hole in the epithelium with an external solution filled pipette. Positive pressure allowed for hole formation as well as separation of OHCs from Deiters' cells. Typically, cells from row 1 (closest to Pillar cells) or row 2 were selected for patching. Cells were recorded from using an internal solution containing (in mM): 116 CsCl, 3.5 $MgCl_2$, 3.5 ATP, 5 creatine phosphate, 1 Tetra Cesium BAPTA (1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N′*,*N′*-tetraacetic acid), 10 HEPES, and 20 ascorbic acid, pH of 7.2 and osmolality of 285 - 290 mOsm.

Whole-cell voltage-clamp was obtained using an axopatch 200b amplifier (Molecular Devices) that was coupled to a computer with the Iotech 3000 DAQ system. JClamp (SCiSoft) controlled data acquisition as well as cell stimulation protocols. Cells were corrected for uncompensated series resistance and junction potential off-line. Cells were discarded if they had more than 50 pA of leak and if mechanotransduction currents were not greater than 800 pA. Dose response curves were obtained for each drug starting from the lowest dose and

incrementing to higher doses after running Standard current-displacement protocols as well as current voltage protocols (see examples in text). Drugs were applied for a minimum of two minutes prior to running stimulus protocols. Drug application was not started until baseline properties had equilibrated, typically 5-10 minutes after establishing the whole-cell mode. Data were analyzed using JClamp software or else exported to Excel or OriginPro2018 (Microcal). Dose response curves were fit with a Hill equation: $I=Imax(x^n/k^n+x^n)$ where x is the drug concentration k is the half blocking dose and n is the Hill coefficient. For all these fits n was equal to 1.

Supplemental Figure 1. Development of a novel purification approach that takes advantage of the variance in gentamicin composition.

Supplemental Figure 2. Validation of compounds using ^{13}C NMR.

Supplemental Figure 3. Antimicrobial potency of hospital gentamicin and five C-subtypes.

Supplemental Figure 4. Antimicrobial potency of hospital gentamicin and five individual impurities.

Supplementary Figure 5. Gentamicin subtypes bind and block the hair cell (MET) mechanotransducer channel.

Supplemental Figure 6. Antimicrobial breadth and potency of hospital gentamicin, Mix 1, Mix 2 and C2b.

Supplemental Table 1. Details of the individual gentamicin components.

Supplemental Table 2. Validation of individual components using ^{1}H NMR.

Supplementary Table 3. The number of cochleae used for each drug tested.

Supplemental Figure Legends

Supplemental Figure 1. Development of a novel purification approach that takes advantage of the variance in gentamicin composition. (**A**) Gentamicin subtypes are produced by the *Micromonospora* species. The first 3-ringed gentamicin compound in this pathway is gentamicin A, and there are 2 main pathways for gentamicin C-subtypes synthesis that separates at gentamicin X. Gentamicin components studied are highlighted in black boxes. (**B**) HPLC traces showing relative abundance and order of elution of gentamicin subtypes from five vendors. The subtypes found in gentamicin mixtures vary depending on the *Micromonospora* strain and reaction conditions used. Lots from vendors B and E (pink traces) were used for the purification of the C-subtypes. (**C**) HPLC traces of purified C-subtypes. Purified gentamicin C-subtypes were additionally validated using NMR (see Fig. S2, Table S2). Co-injection of the lots from vendors B and E confirms the identity of the smallest peak as gentamicin C2b in (**D**).

Supplemental Figure 2. Validation of compounds using ^{13}C NMR. **(A)** Gentamicin chemical structure showing the sites of each carbon color coded by ring**. (B)** The differences between individual gentamicin components occur at 6 different sites, indicated by R groups on Ring I. **(C)** ^{13}C spectral data for eight compounds in deuterium oxide. We detected the correct number of carbons for each compound tested, the chemical formulae and number of carbons detected shown at the bottom of the table. Data shows that the differences between compounds induce changes on Ring I structure only, shown in heat map form. Carbon assignments made following data published (1, 2).

Supplemental Figure 3. Antimicrobial potency of hospital gentamicin and five C-subtypes. **(A)** Antimicrobial activity against 15 strains with known resistance to other antibiotics. Black = *S. aureus*, Green = *E.coli*, Blue = *K. pneumoniae*, Brown = *P. aeruginosa*. Each individual strain has a different symbol. **(B)** Antimicrobial activity against 25 wild-type strains.

Supplemental Figure 4. Antimicrobial potency of hospital gentamicin and five individual impurities. **(A)** Antimicrobial activity of hospital gentamicin, sisomicin, gentamicin A, B, X, and G418 against 15 resistant strains. Black = *S. aureus*, Green = *E.coli*, Blue = *K. pneumoniae*, Brown = *P. aeruginosa*. **(B)** Antimicrobial activity against 25 wild-type strains.

Supplementary Figure 5. Gentamicin subtypes bind and block the hair cell mechanotransducer channel. (**A**) Summary Voltage (mV) – Current (nA) plots for gentamicin C1a, C2, C2a and C1 where color denotes drug concentration as indicated. (**B**) Data were derived from the corresponding plot in (A) for each drug. Data shows drug block of MET channel normalized at each voltage. n=8 for each drug.

Supplemental Figure 6. Antimicrobial breadth and potency of hospital gentamicin, Mix 1, Mix 2 and C2b. (A) The breadth of hospital gentamicin and Mix 1 are similar (1 strain difference). **(B)** The potency of hospital and mix 1 are also similar ($P>0.05$, Mann-Whitney U-test) among the different subgroups of *S. aureus* (n=10)*, E. coli* (n=10)*, K. pneumoniae* (n=10)*, P. aeruginosa* (n=10). Included were resistant (15 strains) and non-resistant strains (25 strains). **(C)** The breadth of Mix 1 and Mix 2 are similar (no strain is different). **(D)** There is no difference in potency between Mix 1 and Mix 2 among different subgroups. **(E)** There is one strain difference between Mix 2 and C2b. **(F)** Mix 2 is more potent than C2b against *S. aureus*, and non-resistant strain groups only ($P<0.05$, Mann-Whitney U-test; n=10, n=25).

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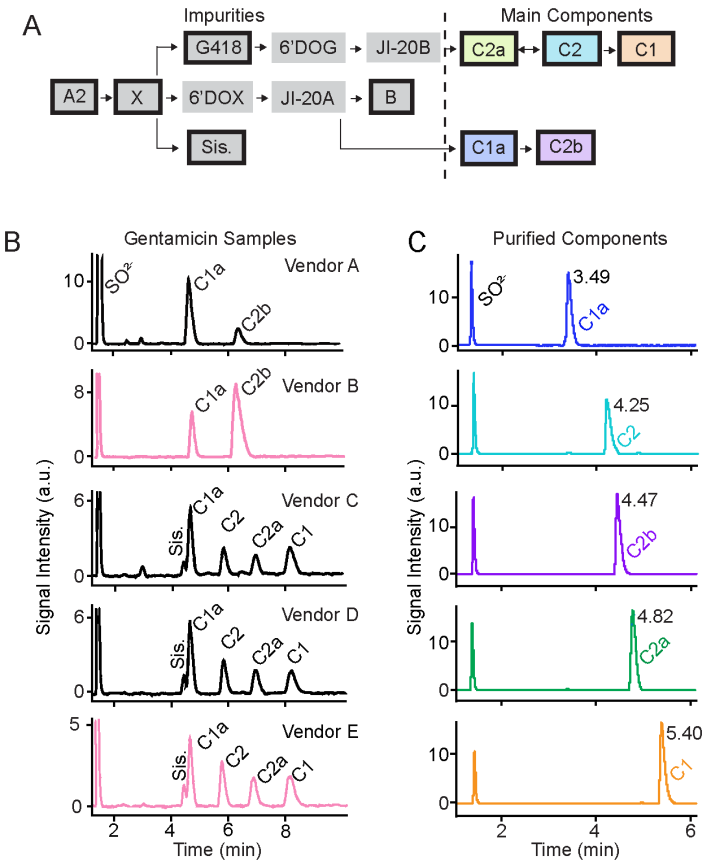
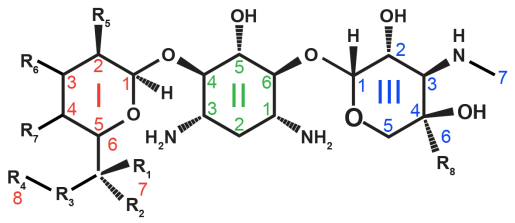

A
Impurities
Main Components
G418
6'DOG
JI-20B
C2a
C2
C1
A2
X
6'DOX
JI-20A
B
Sis.
C1a
C2b
B
Gentamicin Samples
Vendor A
Vendor B
Vendor C
Vendor D
Vendor E
SO4 2-
C1a
C2b
Sis.
C2
C2a
C1
Signal Intensity (a.u.)
Time (min)
C
Purified Components
3.49
4.25
4.47
4.82
5.40

A



B

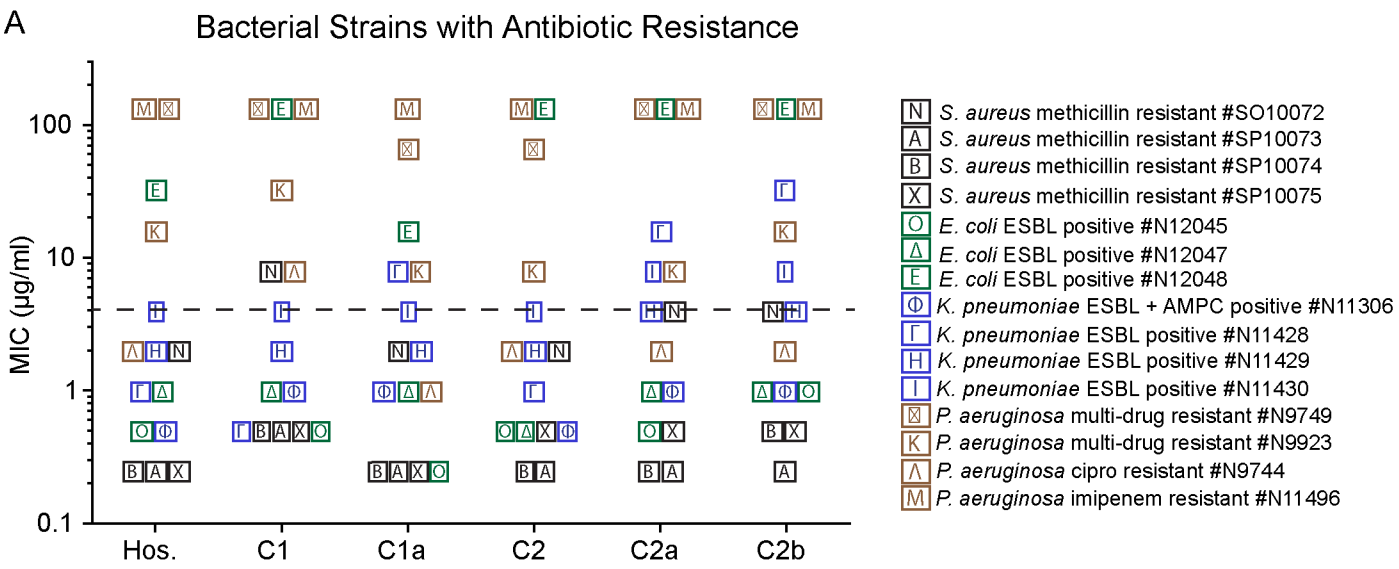
	R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8
Sis.*	-	-	NH_2	-	NH_2	-	-	CH_3
C1a	-	-	NH_2	-	NH_2	-	-	CH_3
C2	CH_3	-	NH_2	-	NH_2	-	-	CH_3
C2a	-	CH_3	NH_2	-	NH_2	-	-	CH_3
C2b	-	-	NH	CH_3	NH_2	-	-	CH_3
C1	CH_3	-	NH	CH_3	NH_2	-	-	CH_3
G418	-	CH_3	OH	-	NH_2	OH	OH	CH_3
A	-	-	OH	-	NH_2	OH	OH	-
B	-	-	NH_2	-	OH	OH	OH	CH_3

* Double bond between atom C4' and C5'

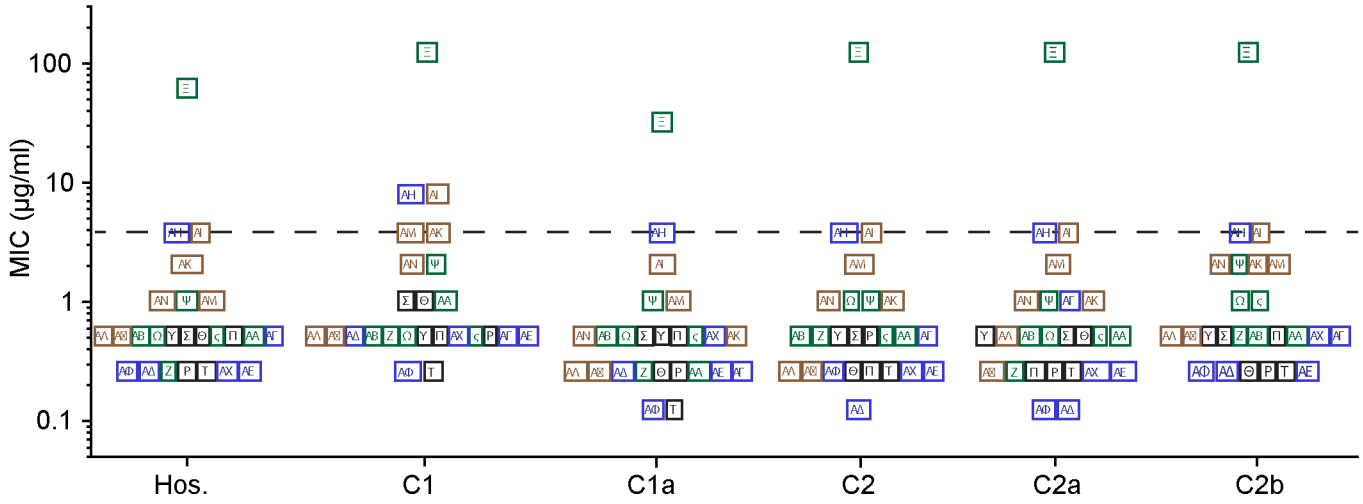
C

Site	Ring	Sisomicin	C1a	C2	C2a	C2b	C1	G418	B	A
1	III	97.22	94.311	94.739	94.042	94.082	94.571	97.441	95.906	96.8
2	III	66.306	66.246	66.238	66.246	66.25	66.246	66.206	66.29	66.25
3	III	63.401	63.293	63.285	63.289	63.289	63.293	63.341	63.365	63.44
4	III	69.917	69.885	69.881	69.889	69.885	69.885	69.881	69.85	69.4
5	III	67.621	67.785	67.793	67.797	67.789	67.793	67.713	67.693	68.97
6	III	20.869	20.877	20.873	20.881	20.877	20.877	20.879	20.837	
7	III	34.483	34.459	34.463	34.459	34.451	34.459	34.487	34.411	29.51
1	II	49.96	49.723	49.687	49.723	49.731	49.711	49.527	49.755	49.53
2	II	27.378	27.626	27.626	27.197	27.606	27.622	28.027	27.522	27.83
3	II	48.132	48.541	48.581	48.641	48.557	48.573	48.91	47.551	48.71
4 or 6	II	83.058	83.547	83.583	83.547	83.555	83.587	83.302	83.863	83.49
5	II	73.592	74.434	74.421	74.55	74.466	74.438	73.572	72.322	73.64
4 or 6	II	79.018	76.02	76.165	75.588	75.7	75.932	75.203	79.755	73.78
1	I	101.168	101.159	101.155	101.176	101.176	101.168	100.951	101.232	100.61
2	I	46.044	48.617	48.749	48.641	48.557	48.685	54.136	70.691	60.64
3	I	23.639	25.47	22.889	25.197	25.546	23.086	70.125	70.691	60.36
4	I	100.338	20.437	20.545	20.212	20.38	20.549	81.687	72.125	80.13
5	I	143.607	65.998	68.767	70.093	65.657	69.136	69.444	68.707	62.37
6	I	40.57	42.626	49.483	51.082	52.184	57.458	65.244	40.222	53.97
7	I			12.241	14.105		9.287	14.381		
8	I					33.453	31.064			
Chemical Formula		$C_{19}H_{37}N_5O_7$	$C_{19}H_{39}N_5O_7$	$C_{20}H_{41}N_5O_7$	$C_{20}H_{41}N_5O_7$	$C_{20}H_{41}N_5O_7$	$C_{21}H_{43}N_5O_7$	$C_{20}H_{40}N_4O_{10}$	$C_{19}H_{38}N_4O_{10}$	$C_{18}H_{36}N_4O_{10}$
No of Carbons:		19	19	20	20	20	21	20	19	18

A

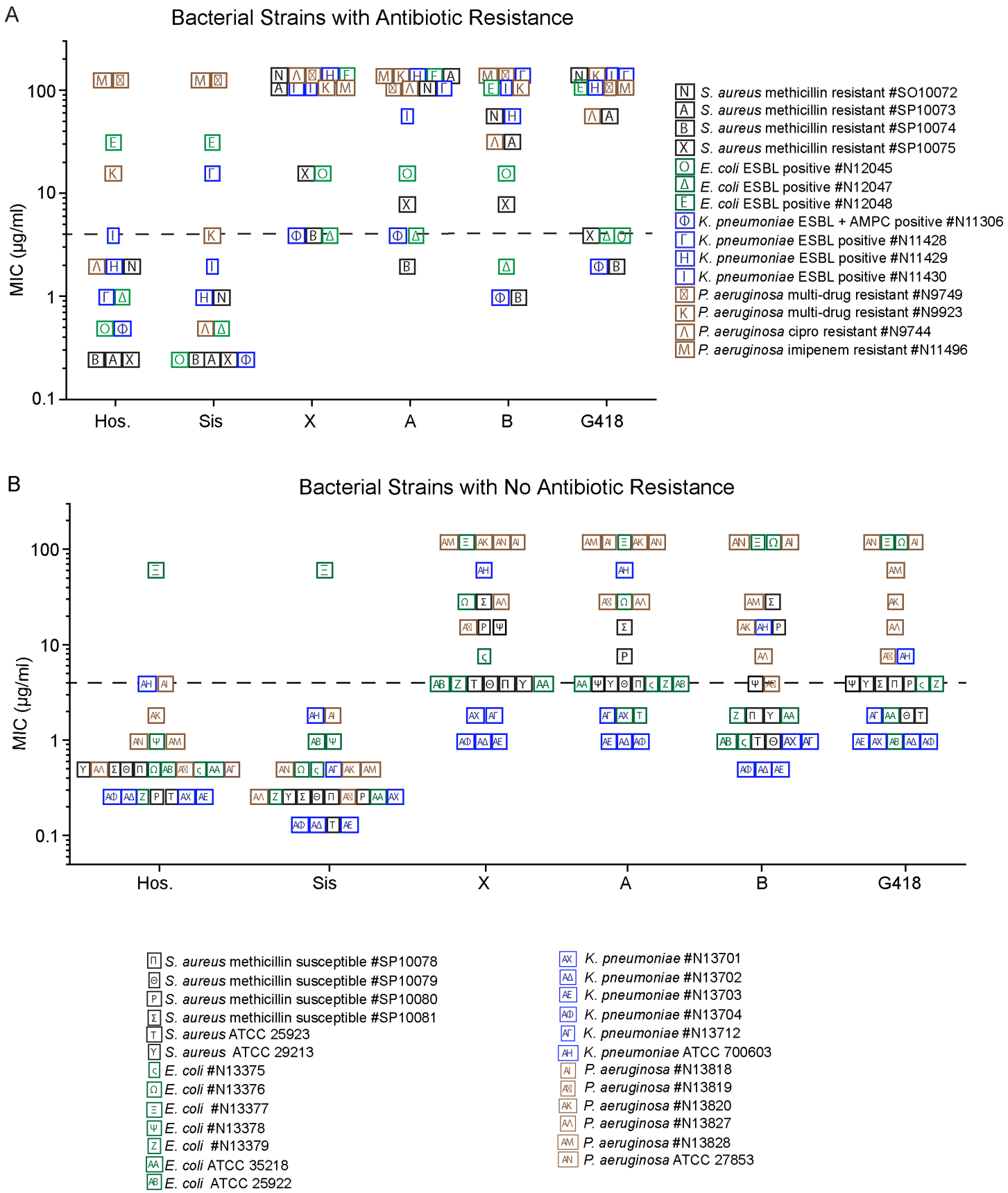


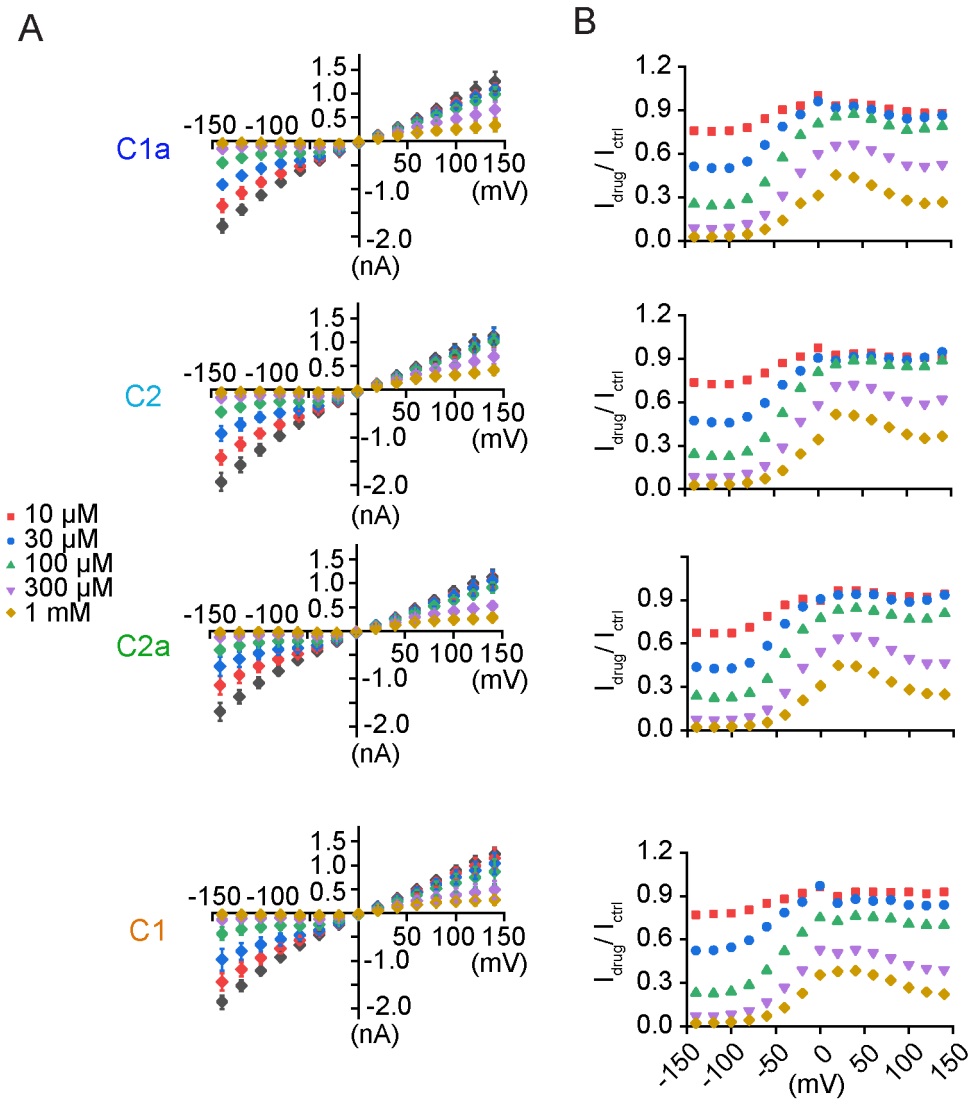
B

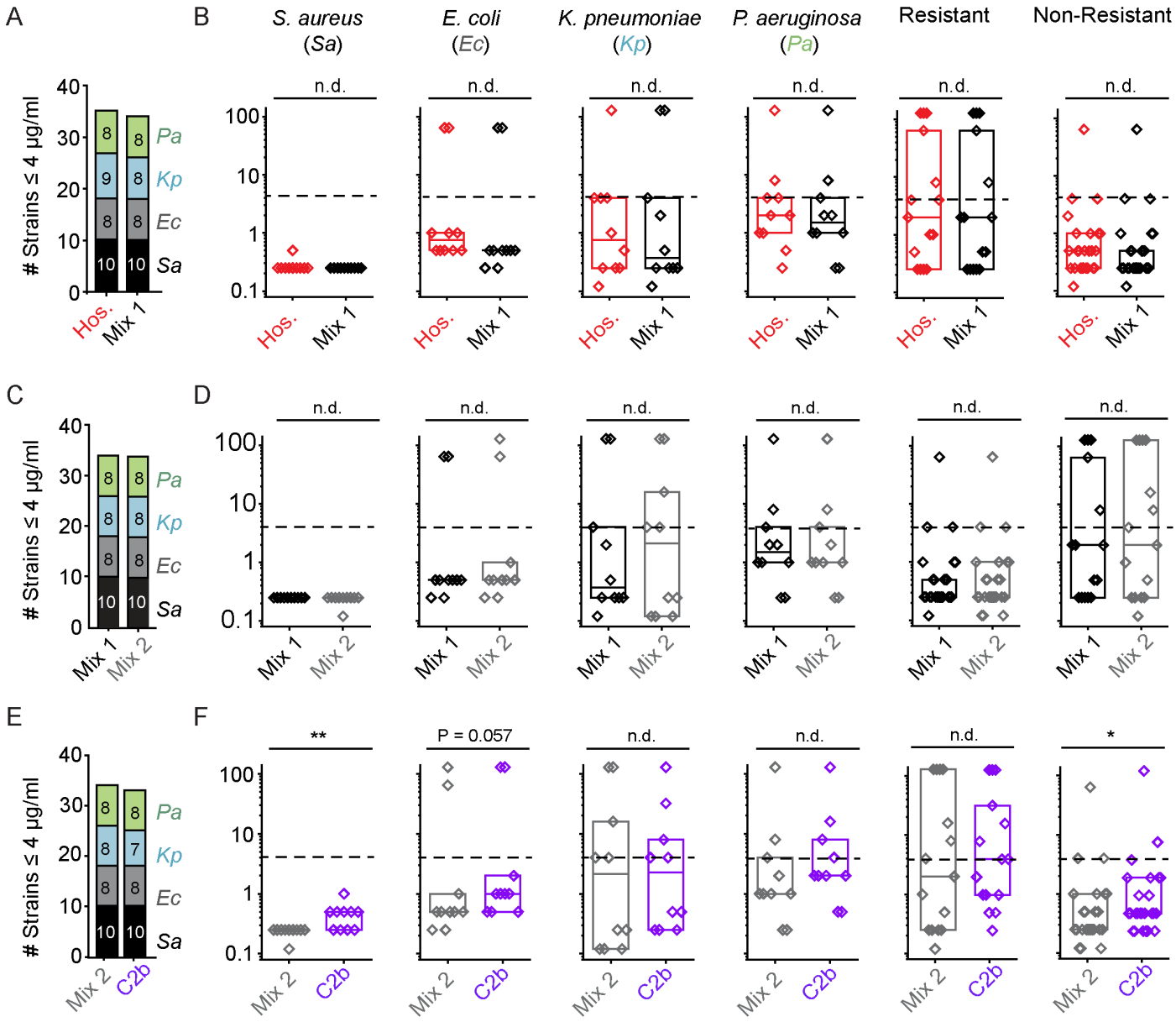


Π S. aureus methicillin susceptible #SP10078
Θ S. aureus methicillin susceptible #SP10079
P S. aureus methicillin susceptible #SP10080
Σ S. aureus methicillin susceptible #SP10081
T S. aureus ATCC 25923
Y S. aureus ATCC 29213
ς E. coli #N13375
Ω E. coli #N13376
Ξ E. coli #N13377
Ψ E. coli #N13378
Z E. coli #N13379
AA E. coli ATCC 35218
AB E. coli ATCC 25922

AX K. pneumoniae #N13701
AΔ K. pneumoniae #N13702
AE K. pneumoniae #N13703
AΦ K. pneumoniae #N13704
AΓ K. pneumoniae #N13712
AH K. pneumoniae ATCC 700603
AI P. aeruginosa #N13818
AΧ P. aeruginosa #N13819
AK P. aeruginosa #N13820
AΛ P. aeruginosa #N13827
AM P. aeruginosa #N13828
AN P. aeruginosa ATCC 27853


A
Bacterial Strains with Antibiotic Resistance
MIC (µg/ml)
100
10
1
0.1
Hos.
Sis
X
A
B
G418
N S. aureus methicillin resistant #SO10072
A S. aureus methicillin resistant #SP10073
B S. aureus methicillin resistant #SP10074
X S. aureus methicillin resistant #SP10075
O E. coli ESBL positive #N12045
Δ E. coli ESBL positive #N12047
E E. coli ESBL positive #N12048
Φ K. pneumoniae ESBL + AMPC positive #N11306
Γ K. pneumoniae ESBL positive #N11428
H K. pneumoniae ESBL positive #N11429
I K. pneumoniae ESBL positive #N11430
☒ P. aeruginosa multi-drug resistant #N9749
K P. aeruginosa multi-drug resistant #N9923
Λ P. aeruginosa cipro resistant #N9744
M P. aeruginosa imipenem resistant #N11496
B
Bacterial Strains with No Antibiotic Resistance
MIC (µg/ml)
100
10
1
0.1
Hos.
Sis
X
A
B
G418
Π S. aureus methicillin susceptible #SP10078
Θ S. aureus methicillin susceptible #SP10079
P S. aureus methicillin susceptible #SP10080
Σ S. aureus methicillin susceptible #SP10081
T S. aureus ATCC 25923
Y S. aureus ATCC 29213
ς E. coli #N13375
Ω E. coli #N13376
Ξ E. coli #N13377
Ψ E. coli #N13378
Z E. coli #N13379
AA E. coli ATCC 35218
AB E. coli ATCC 25922
AX K. pneumoniae #N13701
AΔ K. pneumoniae #N13702
AE K. pneumoniae #N13703
AΦ K. pneumoniae #N13704
AΓ K. pneumoniae #N13712
AH K. pneumoniae ATCC 700603
AI P. aeruginosa #N13818
A☒ P. aeruginosa #N13819
AK P. aeruginosa #N13820
AΛ P. aeruginosa #N13827
AM P. aeruginosa #N13828
AN P. aeruginosa ATCC 27853


A
B
C1a
C2
C2a
C1
10 μM
30 μM
100 μM
300 μM
1 mM
1.5
1.0
0.5
-150 -100
50 100 150
(mV)
-1.0
-2.0
(nA)
1.2
0.9
0.6
0.3
0.0
I_{drug}/I_{ctrl}
-150 -100 -50 0 50 100 150
(mV)


A
B
S. aureus (Sa)
E. coli (Ec)
K. pneumoniae (Kp)
P. aeruginosa (Pa)
Resistant
Non-Resistant
Strains ≤ 4 µg/ml
Pa
Kp
Ec
Sa
Hos.
Mix 1
n.d.
C
D
Mix 2
E
F
C2b
**
P = 0.057
*

Supplemental Table 1 Details of the individual gentamicin components.

Reagent or Resource	Source	Product #	Vendor Purity	Vendor Method	NMR Performed By
Antibiotic					
Gentamicin C1 Sulfate	Nanosyn Inc	NSN22861	99.90%	ELSD	Nanosyn + Stanford
Gentamicin C1a Sulfate	Nanosyn Inc	NSN22860	99.90%	ELSD	Nanosyn + Stanford
Gentamicin C2 Sulfate	Nanosyn Inc	NSN22863	97.50%	ELSD	Nanosyn + Stanford
Gentamicin C2a Sulfate	Nanosyn Inc	NSN22862	98.70%	ELSD	Nanosyn + Stanford
Gentamicin C2b Sulfate	Nanosyn Inc	NSN22864	99.40%	ELSD	Nanosyn + Stanford
Sisomicin Sulfate	Xian Health China	Batch HB20160505	99%	n/a	Nanosyn + Stanford
Gentamicin B Acetate	MicroCombiChem	MCC3477	98%	ELSD/MS	MicroCombiChem + Stanford
Geneticin Sulfate	Toronto Research Chemicals	G360580	96%	1H NMR + MS	TRC + Stanford
Gentamicin A Sulfate	Toku-E	G035	99.20%	1H NMR + MS	Toku-E + Stanford
Gentamicin X Sulfate	Toku-E	G036	99.20%	1H NMR + MS	Toku-E

Supplemental Table 2. Validation of individual components using ^{1}H NMR.

Sisomicin				Gentamicin C1a				Gentamicin C2				Gentamicin C2a				Gentamicin C2b				Gentamicin C1			
P	M	H	J	P	M	H	J	P	M	H	J	P	M	H	J	P	M	H	J	P	M	H	J
5.62	d	1	1.3	5.87	s	1		5.89	d	1	3.6	5.95	d	1	3.56	5.9	d	1	3.38	5.93	s	1	
5.2	t	1	3.8	5.13	s	1		5.13	d	1	3.9	5.14	d	1	3.8	5.13	d	1	3.7	5.14	s	1	
5.14	d	1	3.8	4.22	d	1	11.4	4.22	d,d	1	11.0, 3.6	4.23	d, d	1	11.0, 3.8	4.23	d, d	1	10.9, 3.8	4.23	d	1	10.8
4.22	dd	1	10.8, 3.8	4.14	t	1	8.6	4.09	d, t	1	12.2, 2.6	4.11	t	1	9.5	4.18	t, t	1	10.0, 2.6	4.16	d	1	12.2
4.14	t	1	9.8	4.08	t	1	8.9	4.03**	m	2		4.02	d	1	12.9	4.11	t	1	9.6	4.07	t	1	9.2
4	d	1	12.8	4.01	d	1	12.5	4.01	d		12.4	3.88	t	1	9.3	4.02	d	1	12.8	4.02	d	1	13.1
3.95	m	1		3.84	m	2	7.4	3.84	d	2		3.86	d	1	7.5	3.8	q	2	6.9	3.86	m	2	
3.87	t	1	9.9	3.56	m	3		3.52-3.64	m	4		3.85	m	1		3.62-3.55	m	2		3.57	m	1	
3.79	t	1	9.5	3.5	s	1		3.5	s	1		3.52-3.64	m	3		3.55	t	1	4.4	3.51	s	3	
3.72	t	1	14.2	3.48	s	1		3.48	d	1	1.9	3.52	d	1	2.4	3.51	d	1	2.8	3.5	s	3	
3.68	d	1	14.2	3.33*	s			3.33*	s			3.5	d	1	4.4	3.49	d	1	4.6	3.45	m	1	
3.58	m	1		3.24	d	1	12.6	2.92	s	3		3.35*	q	1	7.5	3.25	d,d	1	13.1, 2.8	3.34	s	1	
3.54	m	1		3.07	t	1	10.8	2.55	d,t	1	12.5, 4.3	2.93	s	3		3.34				2.93	s	3	
3.5	d	1	10.7	2.91	s	3		2.08	q	1	12.7	2.55	d,t	1	12.5, 4.2	3.12	d,d	1	13.2, 9.1	2.75	s	3	
3.49	d	1	12.6	2.54	d	1	13.3	2.04	m	1		2.15	q	1	12.8	2.92	s	3		2.56	d	1	12.5
2.93	s	3		2.09	q	1	12.4	2.02	m	1		2.03	m	3		2.76	s	3		2.1	q	1	13
2.73	d	1	19.2	2.03	d	1	16.3	1.89	d	1	13.6	1.53	m	1		2.55	d, t	1	12.7, 4.6	2.05	s	1	
2.57	d,t	1	12.4, 4.0	2.01	s	1		1.6	m	1		1.35	s	3		2.13	q	1	12.5	2.04	s	1	
2.39	d	1	19.2	1.93	d	1	14	1.34	s	3		1.32	d	3	6.8	2.06	q,d	1	12.3, 3.8	1.91	d	1	13.1
2.11	q	1	12.3	1.57	q	1	11.7	1.28	d	3	6.6					2.01	m	1	4.1	1.62	m	1	
1.35	s	3		1.34	s	3										1.94	d,q	1	13.9, 3.2	1.35	s	3	
																1.56	q, d	1	12.6, 4.2	1.3	d	1	6.75
																1.35	s	3					
Hydrogen Count:			25				27				29				29				30				33

-^{1}H spectral data for seven components, including peak, multiplicity, hydrogen count and the coupling constant (J) show in hertz.

-P = peak, M = multiplicity, H = hydrogen count, J = coupling constant. The asterisk (*) at 3.33-3.35 in C1a, C2, and C2a indicates an additional peak integrating to 0.6 and the double asterisk (**) at 4.03 in C2 indicates an additional peak integrating to 0.6.

Supplementary Table 3 The number of cochleae used for each drug tested.

	Drug Concentration (μM)												No of Cochleae
Drug	0	30	100	150	200	300	400	500	750	1000	3000	5000	
C1	-	8	8	-	-	8	-	8	8	8	8	-	56
C1a	-	8	8	-	-	8	-		8	8	8	-	48
C2	-	7	-	-	-	8	-	16	8	8	-	-	47
C2a	-	8	8	-	-	8	-	8	8	8	5	-	53
C2b	-	8	8	-	-	8	-	8	-	8	8	-	48
Geneticin	-	8	8	-	-	8	-	6	7	6	11	6	60
Hospital	-	8	8	-	-	8	-	8	8	8	-	-	48
Sisomicin	-	12	24	57	60	42	23	24	-	-	-	-	242
No Drug	67	-	-	-	-	-	-	-	-	-	-	-	67
Mix 1	-	8	8	-	-	8	-	8	8	8	8	-	56
Mix 2	-	8	8	-	-	8	-	8	8	8	8	-	56
Total													783