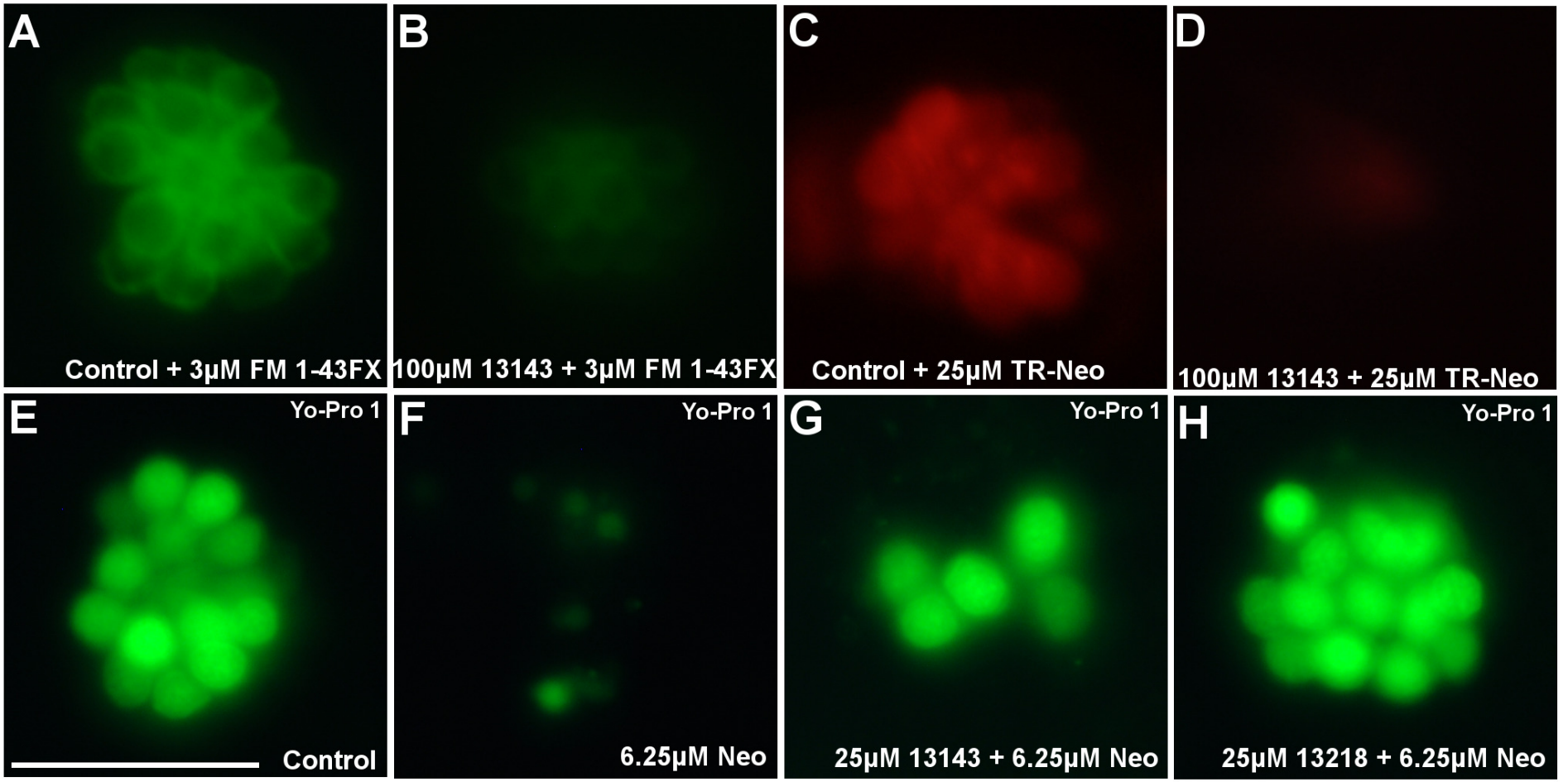
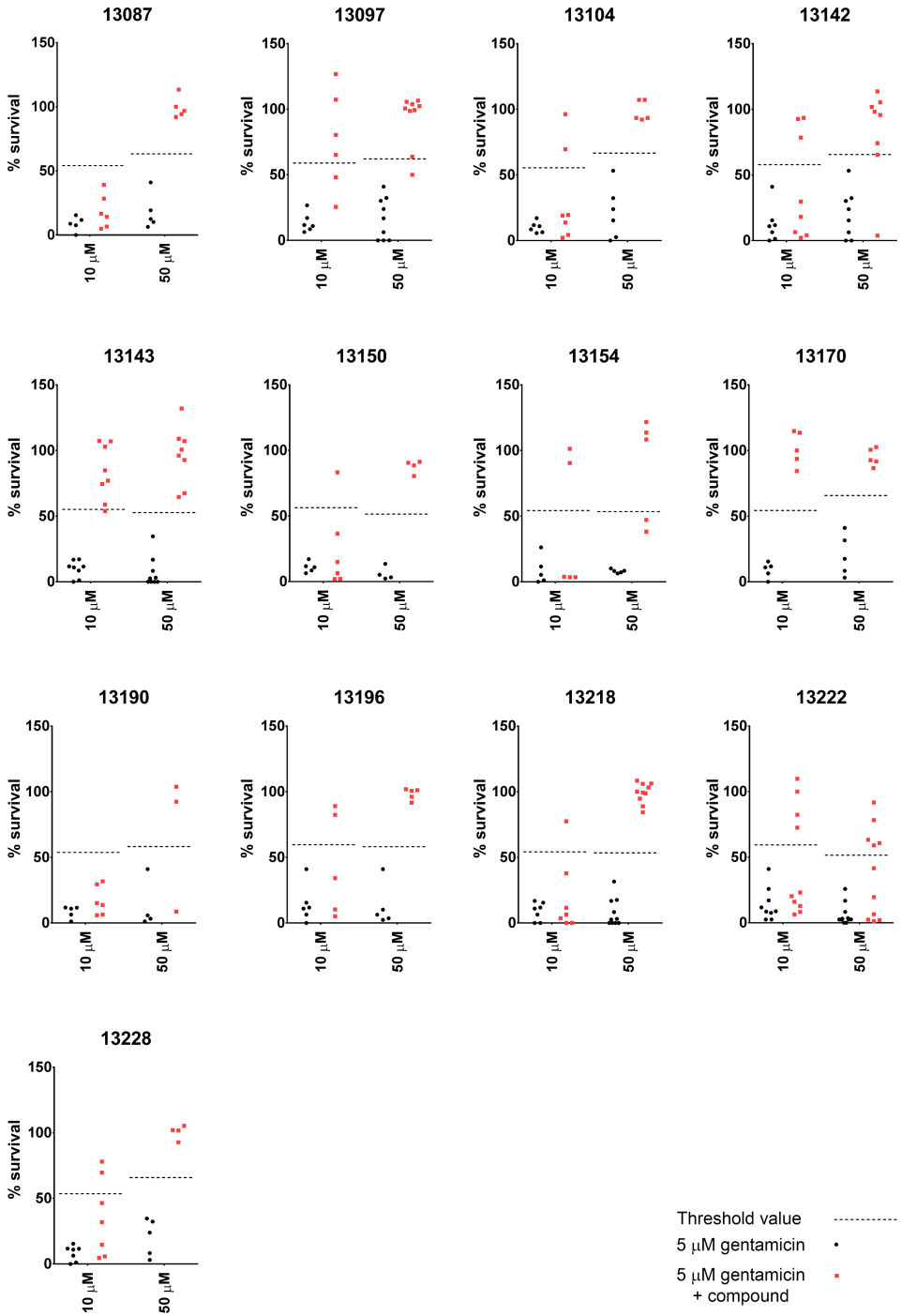


Supplemental Figure 1: Zebrafish assays for potential otoprotective compounds.
(A, B) Assay testing the ability of Tocriscreen library compounds to block FM1-43FX from entering hair cells of zebrafish lateral line neuromasts. (A) Control larvae treated with 3 μM FM1-43FX, (B) larvae treated with 100 μM of comp0und 13143 and 3 μM FM1-43FX. (C,D) Assay testing the ability of compounds to block loading of 25 μM of Texas Red-conjugated neomycin (TR-Neo). (C) Control larvae treated with 25 μM TR-Neo, (D) larvae treated with 100 μM of compound 13143 and 25 μM TR-Neo. (E-H) Assay testing the ability of compounds to protect against neomycin-induced cell death. Hair cells were pre-loaded with 3 μM Yo-Pro-1. (E) Neuromast of a control larva, (F) neuromast of a larva treated with 6.25 μM of neomycin, (G) neuromast of a larva treated with 25 μM of compound 13143 and 6.25 μM of neomycin, (H) neuromast of a larva treated with 25 μM of compound 13218 and 6.25 μM of neomycin. Compound 13218 provides full protection; compound 13143 is partially protective. Representative neuromasts from the trunk (posterior) lateral line are shown in each panel. Images are representative of n = 3 independent experiments with approximately 3 fish per well. Scale bar = 25 μm.



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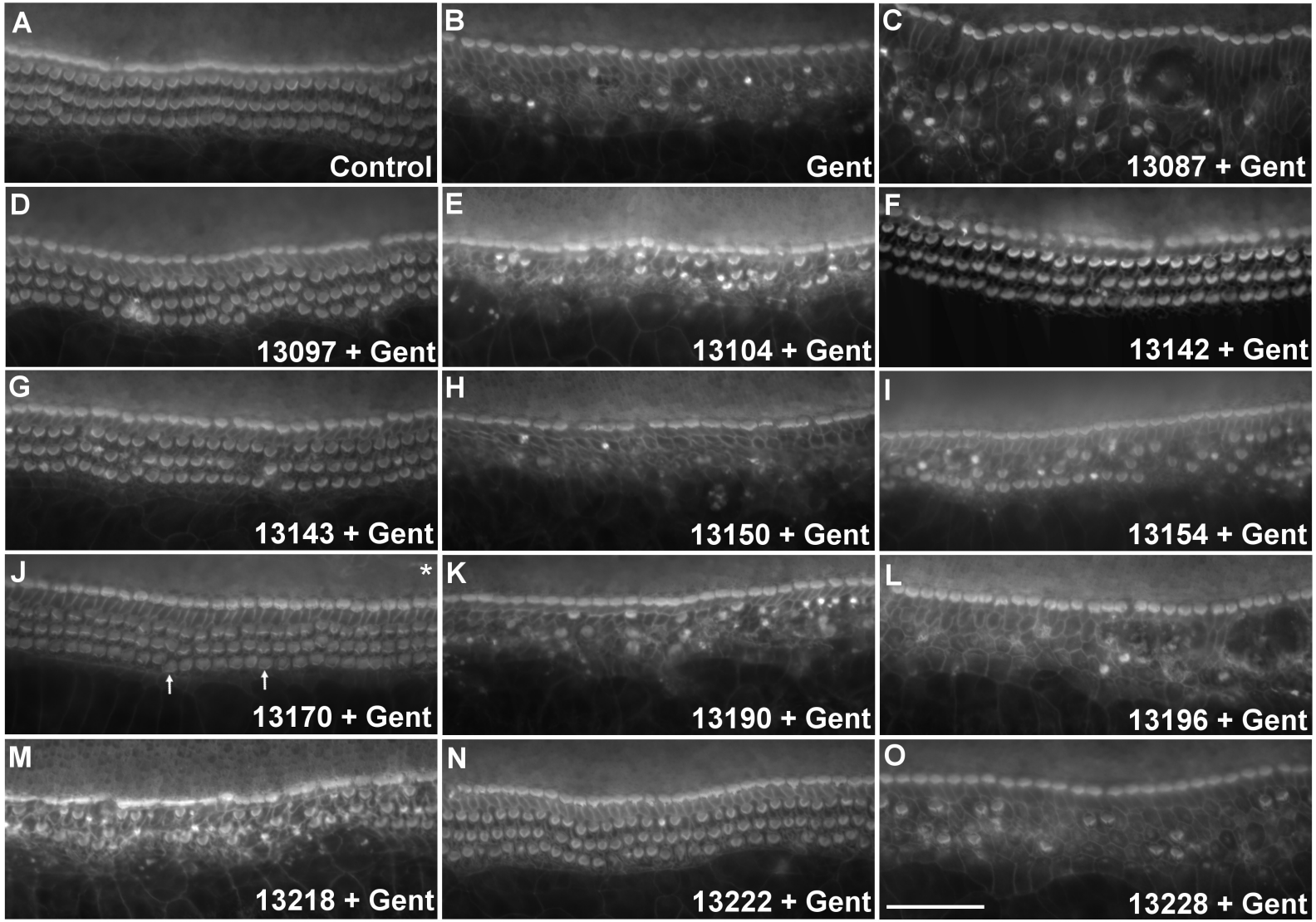
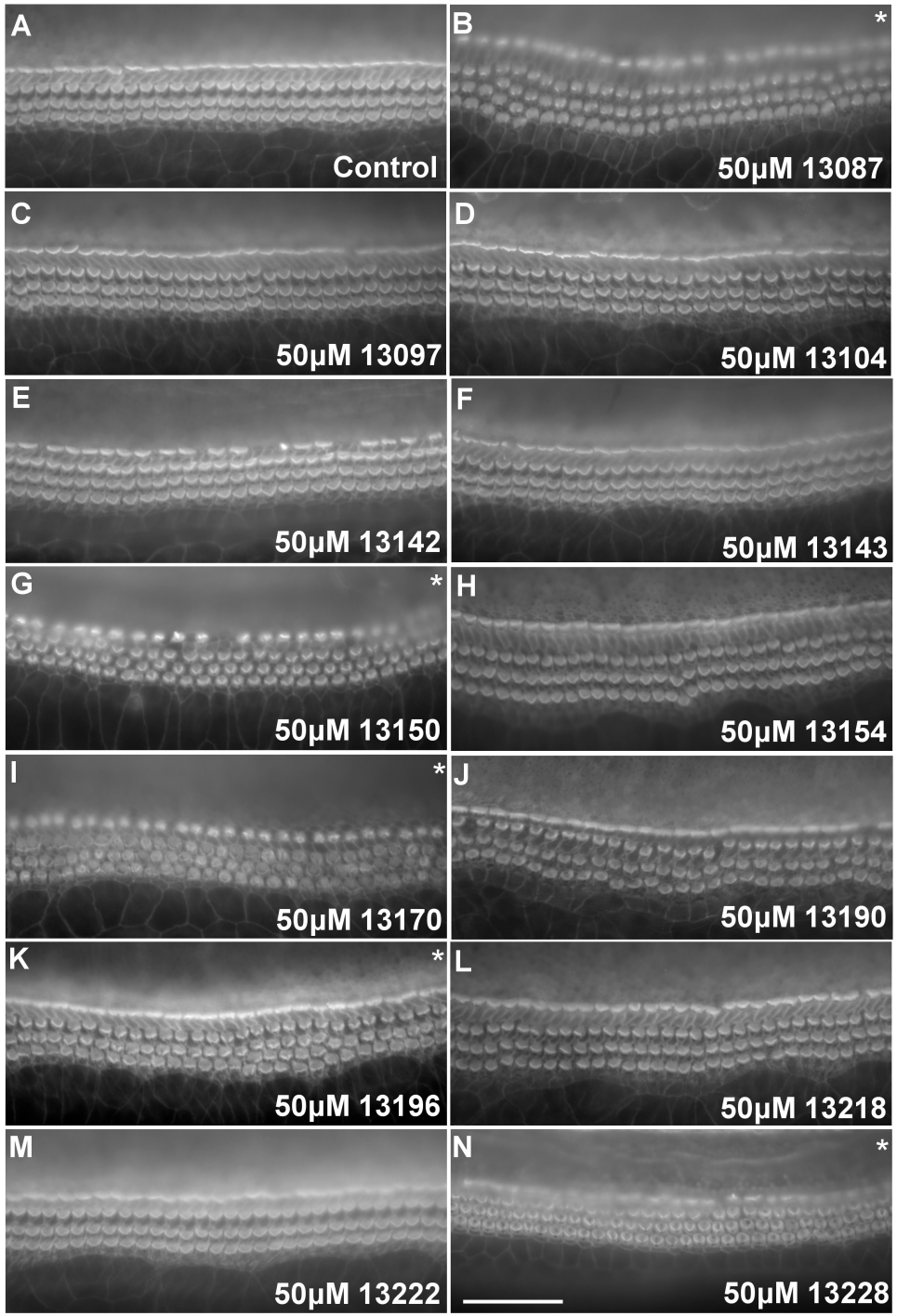


Supplemental Figure 3: Compounds providing protection against gentamicin-induced hair-cell loss in mouse cochlear cultures at a concentration of 10 μM. Cochlear cultures from P2 pups were treated for 48 h with (A) 0.5% DMSO (n = 67), (B) 5 μM gentamicin and 0.5% DMSO (n = 67) or (C-O) 5 μM gentamicin and 10 μM of compounds (C) 13087 (n = 6), (D) 13097 (n = 6), (E) 13104 (n = 7), (F) 13142 (n = 8), (G) 13143 (n = 8), (H) 13150 (n = 6), (I) 13154 (n = 5), (J) 13170 (n = 5), (K) 13190 (n = 6), (L) 13196 (n = 5), (M) 13218 (n = 7), (N) 13222 (n = 10) and (O) 13228 (n = 7). Cultures were labelled with TRITC-phalloidin and images were acquired from the basal coil. Samples are representative. A compound was considered protective if it protected in ≥ 60% of tests. Asterisks identify compounds that damage hair bundles (only compound 13170 in this assay) while arrows indicate specific examples of some of the damaged bundles. Scale bar = 50 μm.



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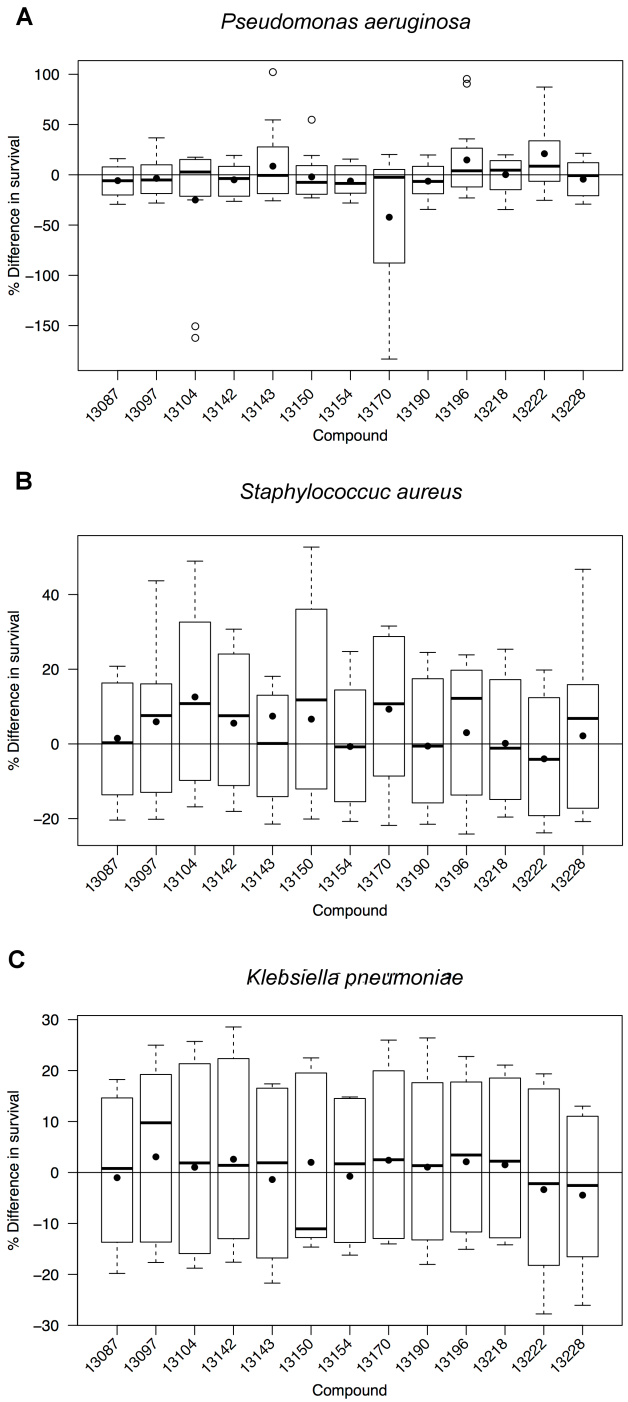
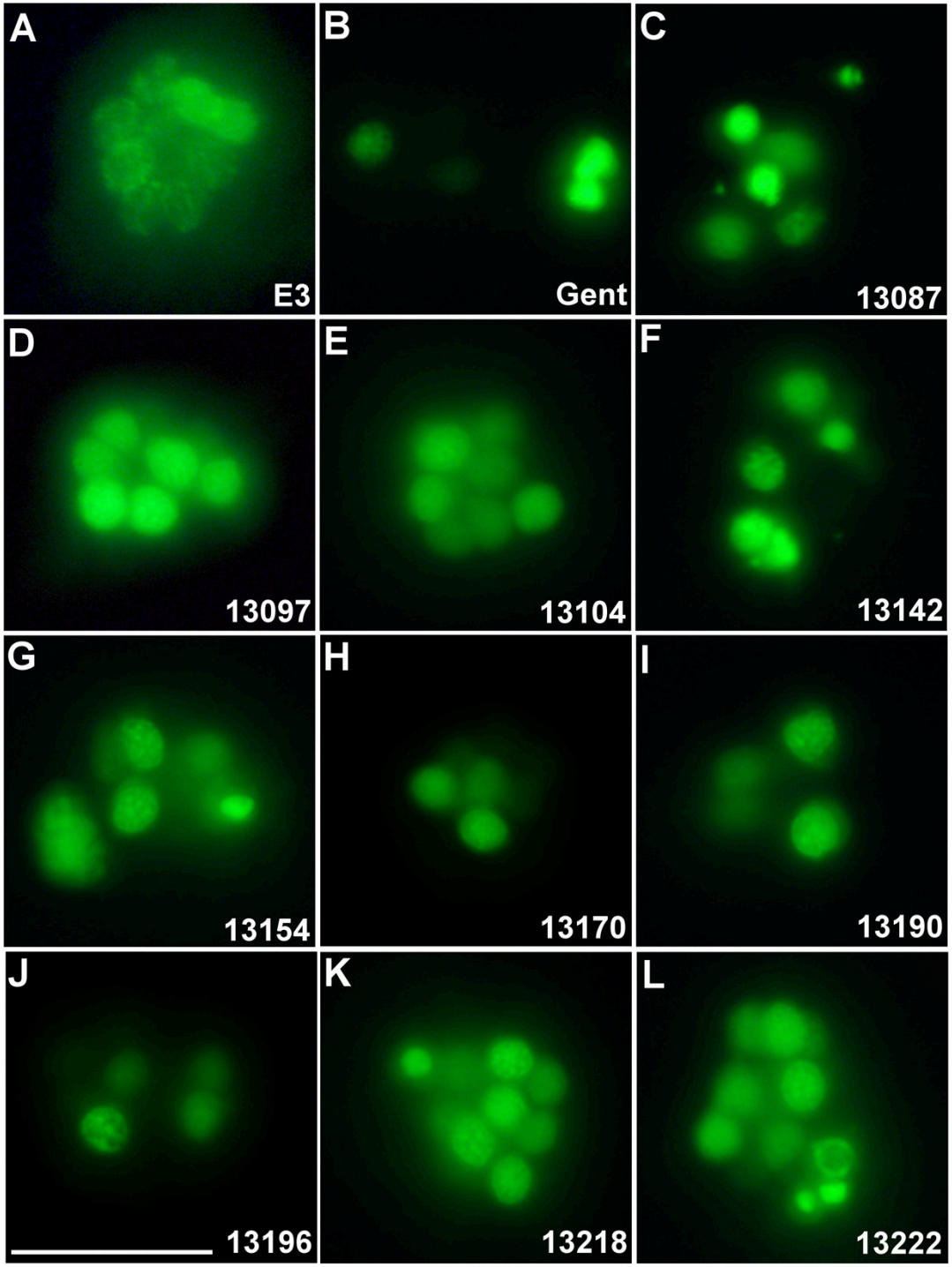


Supplemental Figure 5: Antimicrobial activity of gentamicin in the presence of otoprotective compounds. Box-whisker plots of percentage difference in survival of (A) *Pseudomonas aeruginosa*, (B) *Staphylococcus aureus* and (C) *Klebsiella pneumoniae* (measured by ATP luminescence) in the presence of 2.2 μM gentamicin and 11 μM of the 13 otoprotective compounds (ratio of 1:5) compared to compound-free gentamicin control. Each compound was tested with 3 technical replicates and 3 independent biological replicates. Thick line = median, boxes = interquartile range (IQR), whiskers = an additional 1.5x IQR; means shown as filled circles, outliers shown as open circles.



Supplemental Figure 6: Reduction of gentamicin-induced hair-cell loss in zebrafish larvae with otoprotectants at a concentration of 50 μM. Zebrafish larvae (4 dpf) were treated for 5 h with either E3 control (A), 10 μM gentamicin (B), or (C-L) 10 μM gentamicin and 100 μM of compound as indicated. Neuromasts were pre-labelled with 3 μM Yo-Pro-1. n = 3 independent experiments with 3 or more fish per well. Representative images of individual neuromasts shown. Scale bar = 25 μm.

Supplemental Figures Legends

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UOS#	Mouse culture										Zebrafish	
	Gentamicin Protection (5μM)						Toxicity of Drug Alone		Channel Block		Gentamicin Protection	
	50μM	10μM	5μM	3μM	1μM	0.5μM	100μM	50μM	MET	K+	100 μM	50 μM
13087	5/5	0/6									3/3	2/3
13097	8/9	4/6	3/4	3/4	3/3	3/3					3/3	3/3
13104	5/5	2/7									3/3	3/3
13142	6/8	3/8	1/5								3/3	2/3
13143	8/8	7/8	5/5	3/5	3/4	1/3					LOV	LOV
13150	4/4	1/6									LOV	LOV
13154	3/5	2/5	0/3								3/3	2/3
13170	5/5	5/5									LOV	2/3
13190	2/3	0/6									LOV	2/3
13196	5/5	2/5									3/3	2/3
13218	10/10	1/7									3/3	3/3
13222	5/11	4/10									3/3	3/3
13228	4/4	2/7									LOV	LOV

Key				
	Protection (success rate ≥ 60%)	Ok	Block	Protection
	Partial Protection (success rate > 20% < 60%)	Bad Bundles		Partial Protection
	No protection (success rate ≤ 20%)	Toxic	No Block	No Protection

Supplemental Table 1: Summary of screen data

Table summarizing results of tests performed with mouse cochlear cultures from P2 pups and 4-dpf zebrafish larvae. Data are summarized for the 13 Tocriscreen library compounds that provide protection in mouse cochlear cultures. For the mouse data, numbers indicate the fraction of individual cochlear cultures in which the number of OHCs surviving was greater than the defined threshold (see Supplementary Methods). For the zebrafish gentamicin-protection data, numbers in the cells indicate fraction of independent screens in which the neuromasts were fully protected (green, scored on average 0 - 1) or partially protected (yellow, scored on average > 1 - 2). LOV = loss of larval viability during the assay.

TOCRIS TOP 13	$V_{1/2}$ (mV)	$V_{1/2}$ DEVIATION FROM CONTROL (mV)	S (mV)	S DEVIATION FROM CONTROL (mV)	G_{max} (% OF CONTROL)	G_{max} p	n
13087	-26.2 ± 1.55	1.66 ± 1.40	6.64 ± 0.75	-1.68 ± 0.81	54.5 ± 5.74	0.0014 **	5
13097	-30.5 ± 1.38	-2.64 ± 1.00	6.64 ± 0.48	-1.68 ± 0.36**	61.5 ± 2.58	0.0001***	5
13104	-26.6 ±0.94	3.54 ± 0.83**	4.60 ± 0.41	-2.27 ± 0.51**	48.8 ± 2.41	0.0001***	7
13142	-30.4 ± 0.64	0.98 ± 0.85	5.28 ± 0.43	-1.96 ± 0.72*	62.0 ± 5.17	0.0007***	6
13143	-29.4 ± 0.44	-1.05 ± 0.66	7.44 ± 0.35	-0.26 ± 0.20	76.4 ± 3.50	0.0005***	7
13150	-48.5 ± 1.40	-17.1 ± 1.55**	6.01 ± 1.11	-0.39 ± 0.66	10.0 ± 4.01	0.0283*	2
13154	-28.7 ± 0.36	1.43 ± 0.43*	6.32 ± 0.32	-0.14 ± 0.65	65.7 ± 2.91	0.0013**	4
13170	-30.5 ± 1.94	1.30 ± 0.83	6.70 ± 0.42	-0.24 ± 0.25	104 ± 5.50	0.5155ns	4
13190	-29.1 ± 1.88	1.46 ± 1.01	7.77 ± 0.87	0.19 ± 0.70	79.3 ± 7.11	0.0439*	5
13196	-30.1 ± 1.51	0.45 ± 0.61	6.91 ± 0.44	-0.67 ± 0.39	106 ± 4.50	0.234ns	5
13218	-31.9 ± 1.90	-0.93 ± 0.71	3.37 ± 0.05	-3.97 ± 0.12*	18.9 ± 2.18	0.0169*	2
13222	-30.0 ± 1.32	2.27 ± 0.49**	6.45 ± 0.69	-0.29 ± 0.28	72.9 ± 1.50	0.0001***	5
13228	-19.1 ± 1.49	12.3 ± 2.04*	5.65 ± 0.78	-0.75 ± 0.21	58.7 ± 5.92	0.0199*	3

Supplemental Table 2: Potassium current activation curve data for the otoprotective compounds

Average potentials at which the conductance is half activated ($V_{1/2}$) and voltage sensitivities of activation (S) are shown for each of the 13 compounds found to provide protection in mouse cochlear cultures. The deviation of $V_{1/2}$ from the control value is shown as a positive value for depolarizing shifts and a negative value for hyperpolarizing shifts. Statistically significant shifts were seen for five compounds. The deviation of voltage sensitivity (S) from the control value is shown as a positive value for reduced sensitivity (i.e. greater value of S) and a negative value for increased sensitivity (smaller value of S). Statistically significant changes in sensitivity were seen for four compounds. The maximum conductance (G_{max}) for each compound is shown as a percentage of the control value. All compounds, except for 13170 and 13196, were found to reduce G_{max} by > 20 %. All values are mean ± SEM. Significance was shown by one sample t-test (2-tailed testing) where * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

Supplemental Methods

Analysis of hair-cell survival (protection) in mouse cochlear cultures

Compounds that provided protection were each re-tested on 3-11 individual cochlear cultures. Images of phalloidin-labelled hair cells were captured using a 40x objective (0.75 NA) on a

Zeiss Axioplan2 microscope from the mid-basal region, at a distance ~20% from the basal (hook) end of the cochlea, using a Spot RT slider digital camera. If the cochlear epithelium was not perfectly flat, images were obtained from multiple focal planes and merged using Adobe Photoshop Creative Cloud (2017 Version) to ensure clear visibility of all cells present in the region. Numbers of OHCs were counted in a 221 μm (1200 pixel) length of the organ of Corti. The inclusion criterion for counting was the presence of a hair bundle, with morphological changes to bundle structure being noted separately. The success or failure of a compound was defined using a threshold. Cell counts from cultures grown in gentamicin alone and gentamicin in the presence of the test compound were normalised to counts from the negative control culture (no compound, no gentamicin, vehicle alone) within the same experiment to generate a value for the percentage survival of basal-coil OHCs. A threshold was produced by finding the mean percentage survival of all the gentamicin control coils for a given drug at a given concentration and adding four standard deviations, the standard deviation being generated from all the gentamicin-alone controls run throughout testing. This method provides a stringent and relevant threshold for individual compounds at each concentration tested that also takes into account variability of gentamicin performance across experiments.

For analysis of hair-cell survival in cultures exposed to augmented levels of extracellular Na^+ or K^+, images were collected with a Jenoptik ProgRes Gryphax digital camera from the mid-basal coil of anti-myosin7a-labelled cultures using a 40x LDA Plan (0.55 NA) objective on a Zeiss AXIO VertA1 inverted microscope. Numbers of surviving outer hair cells were counted in three adjacent 100 μm^2 regions of interest from 3-6 cultures for each condition. For statistical analysis see Statistics section.

Electrophysiology

Organotypic cultures on collagen-coated coverslips were transferred, after 1-2 days in culture, to a microscope chamber which was continuously perfused with normal extracellular solution containing (in mM): 135 NaCl, 5.8 KCl, 1.3 $CaCl_2$, 0.9 $MgCl_2$, 0.7 NaH_2PO_4, 5.6 D-glucose, 10 HEPES-NaOH, 2 sodium pyruvate, with MEM amino acids and vitamins added to a final concentration of 1x from stock concentrates (Fisher Scientific). The pH was adjusted to 7.48 (osmolality ~308 $mOsmol.kg^{-1}$). Organs of Corti were observed with an upright microscope (Olympus) with Nomarski differential interference contrast optics using a 40x water-immersion objective. Whole-cell patch-clamp recordings, in either voltage-clamp or current-

clamp mode, were performed at room temperature (20-23°C) using an Optopatch patch-clamp amplifier (Cairn Research). For MET current recordings patch pipettes (2.5-3 MΩ) contained (in mM): 137 CsCl, 2.5 $MgCl_2$, 1 EGTA-CsOH, 2.5 Na_2ATP, 10 sodium phosphocreatine and 5 HEPES-CsOH, pH 7.3 (osmolality ~295 mOsmol.kg^{-1}). For basolateral potassium current recordings, patch pipettes contained (in mM): 131 KCl, 3 $MgCl_2$, 5 Na_2ATP, 1 EGTA-KOH, 5 HEPES-KOH, 10 sodium phosphocreatine, pH 7.28 (osmolality ~295 mOsmol kg^{-1}). Patch pipettes were coated with surf wax (Mr Zogs SexWax) to minimize the fast capacitance transient across the wall of the patch pipette. MET currents were elicited by stimulating the OHC hair bundles using a fluid jet from a pipette (tip diameter 8-10 μm) driven by a piezoelectric disc (12, 13) (12,13). Mechanical stimuli (filtered at 1.0 kHz, 8-pole Bessel) were applied as 45 Hz sinusoids with driver voltage amplitudes of ±40 V. Currents were acquired using pClamp (Molecular Devices) software and stored on computer for off-line analysis. The test compounds were locally superfused onto the OHCs at a concentration of 30 μM or 50 μM in a control solution containing (in mM): 145 NaCl, 5.8 KCl, 1.3 $CaCl_2$, 0.9 $MgCl_2$, 0.7 NaH_2PO_4, 5.6 glucose, 10 HEPES-NaOH, 2 sodium pyruvate and BSA (0.5 mg.ml^{-1}). The pH was adjusted to 7.48 (osmolality ~305 mOsmol.kg^{-1}). Negative pressure applied to the tip of the fluid jet pipette resulted in the compound-containing solution being sucked into the pipette during superfusion. This prevented any mixing and dilution of the compound with control solution during fluid jet stimulation. MET currents were recorded before, during and after compound exposure at membrane potentials ranging from -164 mV to +96 mV.

Basolateral currents were recorded before, during and after compound exposure at membrane potentials ranging from -154 mV to + 46 mV from a holding potential of -84 mV (this value includes a -4 mV correction for the liquid junction potential between extra- and intracellular solutions). The reduction in size of the conductance contributed by $I_{K,neo}$ was determined from the tail currents upon return of the membrane potential after the voltage steps to a constant value away from the reversal potential V_{rev} (-80 mV), at -44 mV. Tail current size was plotted against membrane potential of the preceding voltage steps for the cells and the data were fitted with a first-order Boltzmann function:

$$I = \frac{I_{max}}{1 + exp\left(\frac{V_{1/2} - V}{S}\right)}$$

where I is the tail current, I_{max} is the maximal tail current, $V_{1/2}$ is the half-activation potential, V is the membrane potential of the preceding voltage steps and S is the voltage sensitivity of

activation. The maximum conductance of $I_{K,neo}$ was calculated from the tail currents as $G_{max} = I_{max}/(V - V_{rev})$.

In voltage-clamp recordings, membrane potentials were not adjusted for small errors (< 5 mV) due to residual series resistance (0.7 - 1.5 MΩ). The resting membrane potential was measured as the zero-current potential in voltage clamp mode and any changes subsequently monitored in current-clamp mode before, during and after exposure to the test compounds.

Bacterial growth conditions and antimicrobial susceptibility testing

Klebsiella pneumoniae, Pseudomonas aeruginosa and *Staphylococcus aureus* (clinical isolates from the Royal Sussex County Hospital, Brighton, UK) were passaged twice on LB agar from frozen stocks for antimicrobial susceptibility testing. Mueller Hinton Broth (MHB) was used for all liquid cultures, incubating at 37°C with shaking at 200 RPM. The MIC of gentamicin was established using a microbroth dilution method (1) to be 2.2 μM for all three strains. To test whether the compounds abrogated antimicrobial efficacy of gentamicin, 1X MIC gentamicin (2.2 μM) was supplemented with each compound at a ratio of 1:5 (gentamicin:compound). Drug combinations were inoculated with 3-5 x 10^5 CFU/mL *K. pneumoniae, P. aeruginosa* or *S. aureus* and incubated at 37°C for 16-20 h before quantifying bacterial cell viability using the BacTiter-Glo™ Microbial Cell Viability Assay (Promega) (2). Each experiment was repeated in triplicate and analysed using linear mixed-effects models, modelling the multiple variance components in this hierarchical experimental design (3). Each bacterial assay (*Klebsiella pneumoniae, Pseudomonas aeruginosa,* and *Staphylococcus aureus*) was analysed separately. For statistical analysis see Statistics section.

1. Clinical and Laboratory Standards Institute. *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard — Tenth ed*. Wayne, PA: Clinical and Laboratory Standards Institute; 2015.

2. Rybniker J, et al. Lansoprazole is an antituberculous prodrug targeting cytochrome bc1. Nat Commun. 2015;6:7659.

3. Pinheiro J, Bates D. *Mixed-Effects Models in S and S-PLUS (Statistics and Computing)*. New York, NY: Springer-Verlag; 2000.