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ORIGINAL PAPER

Profiling drug-induced cell death pathways in the zebrafish lateral line

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Abstract Programmed cell death (PCD) is an important process in development and disease, as it allows the body to rid itself of unwanted or damaged cells. However, PCD pathways can also be activated in otherwise healthy cells. One such case occurs in sensory hair cells of the inner ear following exposure to ototoxic drugs, resulting in hearing loss and/or balance disorders. The intracellular pathways that determine if hair cells die or survive following this or other ototoxic challenges are incompletely understood. We use the larval zebrafish lateral line, an external hair cell-bearing sensory system, as a platform for profiling cell death pathways activated in response to ototoxic stimuli. In this report the importance of each pathway was assessed by screening a custom cell death inhibitor library for instances when pathway inhibition protected hair cells from the aminoglycosides neomycin or gentamicin, or the chemotherapy agent cisplatin. This screen revealed that each ototoxin likely activated a distinct subset of possible cell death pathways. For example, the proteasome inhibitor Z-LLF-CHO protected hair cells from either aminoglycoside or from cisplatin, while D-methionine, an antioxidant, protected hair cells from gentamicin or cisplatin but not from neomycin toxicity. The calpain inhibitor leupeptin primarily protected hair cells from neomycin, as did a Bax channel blocker. Neither caspase inhibition nor protein synthesis inhibition altered the progression of hair cell death. Taken together, these results suggest that ototoxin-treated hair cells die via multiple processes that form an interactive network of cell death signaling cascades.

Keywords Hair cell · Ototoxicity · Neomycin · Gentamicin · Cisplatin

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Introduction

The notion that cells can "deliberately" die via a series of complex, ordered events is a core concept in cell biology [1]. Classical programmed cell death, or apoptosis, is characterized by chromatin condensation and activation of caspases, a family of cysteine proteases [2–4]. Apoptotic pathways are critical for normal development and are also activated in many disease states, including neurodegenerative diseases [5, 6]. Furthermore, the dysfunction of apoptosis is considered a hallmark of cancer [7].

Other forms of cell death such as necrosis were originally considered independent of cell signaling events [8, 9]. Recent studies have demonstrated that programmed cell death (PCD) can occur in the absence of caspase activation, leading to the idea that PCD encompasses a broad range of

signaling pathways, all of which result in the orderly demise of the cell [10–13]. However, our understanding of what pathways are necessary for death due to a specific toxin or class of related toxins is still incomplete.

One system where the features of multiple cell death processes are apparent is in toxicity of sensory hair cells of the inner ear. Aminoglycoside antibiotics, platinum-based chemotherapy agents such as cisplatin, and numerous other chemical, biological and environmental challenges can cause sensory hair cell damage, often resulting in hearing loss. Mounting evidence suggests that there are multiple possible mechanisms by which hair cells may be killed, complicating therapeutic intervention [14–19]. The predominant view of aminoglycoside-induced hair cell death is that the build up of oxygen free radicals is a critical early process that sets in motion a variety of other degradative events [19, 20]. Studies in avian inner ear epithelia and zebrafish lateral line show that mitochondrial swelling and release of cytochrome *c* into the cytoplasm are early signs of aminoglycoside ototoxicity [21–25]. Some researchers have demonstrated that caspase inhibition can protect hair cells from aminoglycoside or cisplatin ototoxicity, suggesting that caspase activation occurs downstream of mitochondrial responses [26–29]. However, in vivo studies in mice suggest that caspase-independent cell death pathways may be necessary for kanamycin or cisplatin ototoxicity [15, 30].

Interpreting these studies is complicated by the many different experimental conditions employed in ototoxicity research, such as in vitro vs. in vivo conditions, choice of specific aminoglycoside or other ototoxin, and dose-dependent differences in cell death responses. We approach the problem of cell death signaling in ototoxicity by using the larval zebrafish (*Danio rerio*) lateral line, an in vivo model where quantitative studies are possible across ototoxins and concentration ranges. This system provides a platform for screening multiple cell death pathway inhibitors in parallel to assess pathway activation due to different ototoxic stimuli. The lateral line is a sensory system comprising clusters of neuromasts arrayed in stereotyped positions on the head and body of the fish [31–33]. Each neuromast contains 10–20 mechanosensory hair cells and associated supporting cells. Fish use this sensory system to detect near-field water movement (within a few body lengths) associated with prey, predators, and conspecifics as well as for orientation behavior in flowing water [34–39].

Hair cells in the zebrafish lateral line are considered homologous to sensory hair cells in the mammalian inner ear and have structural and functional similarities, including similar responses to ototoxic drugs [40–46]. We have previously used the lateral line of larval zebrafish for identifying novel protective compounds by screening libraries of drugs or drug-like molecules [47–50]. These screens have uncovered new small molecule protective compounds as well as identifying potential off-label uses for existing therapeutics. The current study uses a similar chemical genetic approach, but here we used a library of known cell death inhibitors in order to more fully understand the variety of signaling pathways activated by known ototoxins in this system.

We have recently shown that the closely related aminoglycoside antibiotics neomycin and gentamicin appear to elicit distinct, partially overlapping cell death responses in the zebrafish lateral line [46]. Experiments with protective mutants and drugs suggest that activation of an "acute" cell death mechanism is shared by both neomycin and gentamicin, while a "slow" mechanism is specific to gentamicin-induced damage [46, 51]. In contrast, cisplatin-induced hair cell loss appears to be linear and cumulative in zebrafish. In addition, pharmacologic and genetic research in zebrafish and mice suggests that cisplatin and aminoglycosides may activate different signaling pathways [44, 47, 50, 52], The present study profiles pathway activation in ototoxin-treated hair cells by screening a custom cell death inhibitor library. We show that each toxin activates a distinct subset of the possible pathway space, suggesting that a rich, interconnected network of cell death signaling cascades contributes to hair cell death from a single toxin.

Materials and methods

Animals

Wildtype *AB zebrafish were acquired through group mating and raised at 28.5 °C in Petri dishes containing embryo medium according to standard protocols [53]. All experiments described here used 5–6 days post-fertilization (dpf) larvae. All procedures were approved by the University of Washington Animal Care and Use Committee.

Reagents

Neomycin solution (10 mg/ml) and gentamicin solution (50 mg/ml) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Cisplatin solution was acquired from the University of Washington Medical Center pharmacy. All inhibitors making up the custom library were purchased from Calbiochem (now EMD Millipore, Billerica, MA, USA). Other reagent sources are provided in the text describing the use of each reagent.

Library composition and screening

A custom library of 61 pharmacological inhibitors was assembled to encompass known cell death-associated

molecular targets (e.g., caspases, Bax) as well as a variety of molecules such as FUT-175 that were reported to influence cell death in specific tissues or in response to specific cytotoxic stimuli [54]. Table 1 contains a complete list of library compounds used in the present study.

Compounds were purchased in powdered form and dissolved in the appropriate solvent for each compound based on the manufacturer's recommendation (water, DMSO [≤1 %], or ethanol [≤0.1 %]) as shown in Table 1. Final concentrations of each compound were determined based on published literature, and 10 μM was selected as the screen concentration if the literature was highly variable or if there was little published information for the compound in question. Several compounds were lethal to the fish at the initial concentrations tested. For these compounds, additional toxicity testing was performed to determine the highest concentration at which no morbidity was detected, and this concentration was then used for additional screening. Morbidity was defined as abnormal swimming or righting behavior or abnormal morphology such as body curvature. Table 1 shows final screen concentrations, with superscript "a" indicating concentrations that were empirically determined based on toxicity testing.

All experiments were performed at 28.5 °C in defined E2 embryo medium (EM) (1 mM $MgSO_4$, 120 μM KH_2PO_4, 74 μM Na_2HPO_4, 1 mM $CaCl_2$, 500 μM KCl, 15 mM NaCl, and 500 μM $NaHCO_3$ in dH_2O) [53]. Larvae ($n = 7$–12 per group) were placed in custom fish transfer baskets (constructed from modified 50 ml conical tubes with mesh inserts [40]) and pre-treated in a specific inhibitor for 1 h. This pre-treatment period is consistent with similar protective screens [47, 48]. Following inhibitor pretreatment, fish were treated with either 200 μM neomycin, 50 μM gentamicin, or 500 μM cisplatin. Fish treated with neomycin were incubated in ototoxin for 30 min, followed by four rinses in fresh EM and a 60 min recovery period. Fish treated with gentamicin or cisplatin were incubated continuously in ototoxin for 6 h, followed by two rinses in fresh EM and immediate hair cell assessment. The inhibitor was present during the ototoxin exposure period as well. Concentrations and treatment lengths of ototoxins were previously determined to reduce hair cell staining by 80 %, producing comparable degrees of damage so that the magnitude of protection may be directly compared among ototoxins [44–46, 51]. Negative controls were handled identically, including addition of the appropriate solvent (DMSO or ethanol), but no inhibitor or ototoxin was present, while positive control animals received only ototoxin but no inhibitor. Initial screening was performed "blind" as compounds were numbered and there were no a priori reasons to associate particular numbers with hair cell protection.

Hair cell survival was assessed by the relative fluorescent intensity of staining with the mitochondrial potential dye DASPEI (2-(4-(dimethylamino)styryl)-*N*-Ethylpyridinium Iodide). Following acute or continuous treatment, free-swimming larvae were immersed for 15 min in 0.005 % DASPEI (Life Technologies, Carlsbad, CA, USA), rinsed twice in EM, and anesthetized with 0.001 % MS-222 (Sigma-Aldrich). The same 10 head neuromasts in

Table 1 Cell death inhibitor library components

Compound name	Inhibitor family	Solvent	Stock conc.	Screen conc.	CAS #
AEBSF, Hydrochloride	Protease	Water	10 mM	5 μM	30827-99-7
ALLN	Calpain	DMSO	10 mM	10 μM	110044-82-1
Apoptosis inhibitor	Apoptosis	DMSO	10 mM	10 μM	54135-60-3
Bax channel blocker	Bcl2 proteins	DMSO	10 mM	5 μM	54135-60-3
BAY 11-7082	NF-KB	DMSO	10 mM	250 nM[a]	19542-67-7
Bongkrekic acid, triammunium salt	Mitochondria	Ethanol	1 mM	500 nM	1177154-51-6
Calpain inhibitor III	Calpain	DMSO	10 mM	10 μM	88191-84-8
17-DMAG	Heat shock	Water	1 mM	500 nM	467214-21-7
Chymostatin	Protease	DMSO	2 mg/ml	0.5 ug/ml	9076-44-2
Cyclohexamide	Protein synthesis	Ethanol	10 mM	5 μM	66-81-9
Cyclosporin A	Mitochondria	DMSO	10 mM	5 μM	59865-13-3
Dexamethasone	Nitric oxide	DMSO	10 mM	10 μM	50-02-2
JNK inhibitor II	JNK kinase	DMSO	10 mM	10 μM	129-56-6
Necrosis inhibitor, IM-54	Necrosis	DMSO	10 mM	10 μM	861891-50-1
Necrostatin-1	Necrosis	DMSO	10 mM	1 μM	4311-88-0
PARP inhibitor IV, IQD	PARP	DMSO	10 mM	10 μM	5154-02-9
PARP inhibitor XI, DR2313	PARP	Water	10 mM	10 μM	284028-90-6
Pifithrin-alpha	p53	DMSO	10 mM	10 μM	63208-82-2

Table 1 continued

Compound name	Inhibitor family	Solvent	Stock conc.	Screen conc.	CAS #
PPack, dihydrochloride	Protease	10 mM HCl	10 mM	5 μM	142036-63-3
Sulfasalazine	NF-KB	DMSO	10 mM	10 μM	599-79-1
Antipain, dihydrochloride	Protease	DMSO	1 mM	10 μM	37682-72-7
Apoptosis inhibitor II, NS3694	Apoptosis	DMSO	10 mM	25 μM	426834-38-0
Cathepsin inhibitor III	Protease	DMSO	1 mM	10 μM	NA
Cathepsin G inhibitor I	Protease	DMSO	1 mM	10 μM	429676-93-7
E-64 protease inhibitor	Protease	Water	1 mM	10 μM	66701-25-5
Heat shock protein inhibitor I	Heat shock	DMSO	10 mM	10 μM	218924-25-5
Leupeptin, hemisulfate	Protease	Water	10 mM	100 μM	103476-89-7
Luteolin	Oxidative stress	DMSO	10 mM	10 μM	491-70-3
NEMO-binding domain peptide	NF-KB	DMSO	500 μM	10 μM	NA
NF-kB activation inhibitor	NF-KB	DMSO	1 mM	50 nM[a]	545380-34-5
nNOS inhibitor I	Nitric oxide	Water	1 mM	10 μM	NA
Omi/HtrA2 protease inhibitor, Ucf-101	Mitochondria	DMSO	1 mM	500 nM	313649-08-0
Pepstatin A, synthetic	Protease	DMSO	5 mM	1 μM	26305-03-3
Proteasome inhibitor I	Proteasome	DMSO	1 mM	10 μM	NA
Proteasome inhibitor II	Proteasome	DMSO	1 mM	10 μM	NA
Ro106-9920	NF-KB	DMSO	1 mM	1 μM	62645-28-7
Bax-inhibiting peptide, V5	Bcl2 proteins	Water	1 mM	10 μM	NA
Fas/FasL antagonist, Kp7-6	Apoptosis	Water	500 μM	10 μM	NA
FUT-175	Protease	Water	5 mg/ml	18.5 μM	82956-11-4
Caspase inhibitor II, cell permeable	Caspase	DMSO	1 mM	10 μM	NA
Caspase inhibitor III	Caspase	DMSO	1 mM	10 μM	634911-80-1
Ubiquitin aldehyde	Proteasome	DMSO	100 μM	1 μM	NA
CGP-37157	Mitochondria	DMSO	10 mM	1 μM[a]	75450-34-9
Ru360	Mitochondria	Water	1 mM	10 μM	NA
Protein kinase inhibitor, DMAP	JNK kinase	Water	10 mM	10 μM	938-55-6
MEG, hydrochloride	Nitric oxide	Water	10 mM	20 μM	19767-44-3
Granzyme B inhibitor III/caspase 8 II	Granzyme	DMSO	1 mM	10 μM	NA
TNF-alpha inhibitor	Inflammatory	DMSO	10 mM	100 nM[a]	1049741-03-8
Rapamycin	p70 S6 kinase	DMSO	100 μM	100 nM	53123-88-9
Analog of Trichostatin A	HDAC	DMSO	2 mM	1 μM	58880-19-6
Roscovitine	CDK	DMSO	10 mM	10 μM	186692-46-6
PD98059	MEK	DMSO	10 mM	10 μM	167869-21-8
Caspase1 inhibitor IV	Caspase I	DMSO	10 mM	10 μM	154674-81-4
Resveratrol	Oxidative stress	DMSO	20 mM	20 μM	501-36-0
D-methionine[b]	Oxidative stress	Water	250 mM	1 mM	348-67-4
Glutathione[b]	Oxidative stress	Water	100 mM	500 μM	70-18-8
AG1478	EGF	DMSO	10 mM	10 μM	175178-82-2
Thapsigargin	ER calcium release	DMSO	5 mM	500 nM	67526-95-8
Bcl-2 inhibitor	Bcl2 proteins	DMSO	5 mM	50 μM	383860-03-5
U0126	MEK	DMSO	10 mM	500 nM	109511-58-2
3-MA	Autophagy	EM	20 mM	10 mM	5142-23-4

Additional information and original references for these compounds may be found at www.emdmillipore.com

NA not available

[a] Indicates compounds where the screen concentration was empirically determined based on toxicity assays. All other screen concentrations were selected based on published literature, or set to 10 μM when insufficient published data were available

[b] Compound was purchased from Sigma-Aldrich

each fish were examined in a single field of view using a Leica MZFLIII fluorescent stereomicroscope and 50× magnification. Each neuromast was given a score of "2" if the DASPEI label was bright, a "1" if the label was dim, and a "0" if no label was detected. Zebrafish neuromasts develop in highly stereotyped positions [33, 40], giving us confidence that scores of "0" denote neuromasts damaged due to treatment rather than those missing due to developmental events. Each fish therefore received a total score of 0–20. Scores were normalized to controls such that 100 % represents the average score for control animals. Our previous research demonstrates that this scoring method is robust and allows quantitative assessment of hair cell survival, and that DASPEI scores are highly correlated with direct counts of individual hair cells in the same neuromast [44, 51]. DASPEI assessment offers the additional advantage of speed, allowing an experienced researcher to assay 20–30 fish every 15 min.

Inhibitor "hits" (compounds that protected hair cells from one or more ototoxins) were then retested twice with the same concentration of ototoxin and putative protective compound in order to confirm the protective effect. Hits were considered significant if the inhibitor-treated group had at least twice the averaged total DASPEI score of the group treated with ototoxin only.

Dose–response analyses

Dose–response testing was performed for all confirmed hits. In these experiments we attempted to define what we refer to as the "dose–response matrix" for each combination of ototoxin and putative inhibitor of toxicity. For gentamicin, both acute and continuous treatment durations were used as described above because damage caused by gentamicin exposure is known to vary with treatment length [46]. All other ototoxin incubation periods were as described above. First, the concentration of the putative inhibitor was varied by 1–2 orders of magnitude in order to identify the optimal protective concentration. Larvae (10–12 per treatment group) were pretreated for 1 h in one of four test putative inhibitor concentrations, then co-treated with inhibitor and either 200 μM neomycin (acute), 200 μM gentamicin (acute), 100 μM gentamicin (continuous), or 500 μM cisplatin (continuous). Positive control fish were again treated with ototoxin only. Treatment paradigms and assessment with DASPEI were as described above. Additional groups of fish were treated with the same putative inhibitor concentrations alone to determine if the putative inhibitor alone affected hair cell survival.

Initially, inhibitors were only used in combination with the ototoxin(s) to which they had demonstrated protection during the screen. Once the optimal protective concentration was determined (defined as the concentration that provided maximum hair cell protection with minimal fish toxicity), that concentration was used in a second set of dose–response experiments. Here, a single inhibitor concentration was used and the ototoxin concentrations were varied to encompass a wide range of hair cell damage. For these second stage experiments, all of our ototoxins were used at multiple concentrations (50–400 μM neomycin or gentamicin, 250–1000 μM cisplatin). Again, both acute and continuous treatment paradigms were used for gentamicin experiments, while neomycin experiments were performed only with the acute treatment paradigm, and cisplatin experiments with the continuous exposure paradigm. While these experiments were not performed "blind" with regards to inhibitor treatment, we have found no difference in DASPEI scores in side-by-side comparisons of blinded versus unblinded experiments (Coffin, unpublished data).

Hair cell counts

DASPEI intensity is dependent on mitochondrial membrane potential and some of the inhibitors used in the present study could possibly decouple mitochondrial membrane potential from hair cell survival. To confirm that the DASPEI scoring was accurately predicting hair cell survival, direct counts of labeled hair cells were performed using immunofluorescence. Larvae (8–10 per group) were treated as described above for dose–response analyses, but only a single combination of ototoxin and inhibitor was used. Hair cell counts were performed for each inhibitor that appeared protective based on the DASPEI scoring assays.

After treatment, fish were euthanized in an ice-water bath and fixed in 4 % paraformaldehyde in phosphate-buffer saline (PBS). Fish were rinsed twice in fresh PBS and once in distilled H_2O to improve antibody penetration, then blocked in PBS containing 0.1 % Triton-X and 5 % normal goat serum (both from Sigma-Aldrich). Hair cells were labeled with mouse anti-parvalbumin (EMD Millipore, diluted 1:500 in PBS with 1 % normal goat serum) overnight at 4 °C, rinsed in fresh PBS, and visualized with goat anti-mouse secondary antibody (Alexa Fluor 488 or 568, Life Technologies, diluted 1:500 in PBS). Lateral line neuromasts were viewed on a Zeiss Axioplan 2ie epifluorescent microscope with a 40× objective (NA = 0.75). Hair cell counts were performed in seven neuromasts per fish (SO1, SO2, IO1, IO2, IO3, OP1, and M2; [33]) and counts were summed to arrive at one value per fish. These neuromasts were selected because they are readily viewed when the fish is positioned on bridged glass coverslips, and because six of these neuromasts are also assessed for DASPEI scoring. Previous reports suggest that different neuromasts exhibit identical ototoxic responses at this age [40]. Images were taken of representative neuromasts using

Slidebook software v. 4 (Intelligent Imaging Innovations, Denver, CO, USA), or on an Olympus FV1000 confocal system with associated Fluoview software.

Gentamicin uptake assay

While each inhibitor in the library was selected because it had a known mechanism of intracellular action, it is possible that some inhibitors act on hair cells by attenuating ototoxin uptake. To test this possibility we performed an uptake assay. Fish were incubated for 1 h in the optimal inhibitor concentration, followed by a 10-minute co-incubation in inhibitor and 100 μM gentamicin tagged with the fluorophore Texas red (GTTR; [55, 56]). Excess fluorophore was removed with two rinses in fresh EM and fish were anesthetized with MS-222 and viewed using either an Olympus Fluoview FV1000 confocal microscope or a Leica DMRB fluorescent compound microscope. Neuromast GTTR intensity was assessed qualitatively [51]. In some cases the experimenter was blind to the inhibitor used in conjunction with GTTR.

Caspase inhibition

Multiple caspase inhibitors were included in the initial inhibitor library (see Table 1). We also conducted additional experiments with the pan-caspase inhibitor Z-VAD-Fmk (EMD Millipore). Fish were pre-treated for 1 h in 10–300 μM Z-VAD, a concentration range shown to prevent caspase inhibition in previous zebrafish studies [57, 58]. Fish were then co-treated with Z-VAD and neomycin or gentamicin as described above and hair cell survival was assessed with DASPEI scoring.

Protein synthesis inhibition

PCD requires synthesis of new protein in some circumstances, although in others translational inhibition can promote cell death [59–61]. To examine this issue in the lateral line, we performed additional experiments with the translation inhibitor cycloheximide (EMD Millipore). Fish were pre-treated for 1 h in cycloheximide (1–100 μM), then co-treated with cycloheximide and neomycin, gentamicin, or cisplatin as described for dose–response analyses. Hair cell survival was assessed with DASPEI scoring.

Data analysis

The results from dose–response experiments were analyzed using 1-way or 2-way ANOVA in Prism (v. 5). Bonferroni-corrected posthoc testing was performed if the ANOVA was significant ($p < 0.05$). All data are presented as mean ± 1 SD.

Results

Screening of a custom cell death inhibitor library revealed that different inhibitors protected hair cells from different ototoxins, suggesting that each ototoxin activates a distinct set of cell death pathways (Fig. 1). Figure 1a shows initial screening results using neomycin as the example ototoxin, with 20 compounds initially yielding hair cell protection (bars above the red line). Comparison of initial screen "hits" for each of the three ototoxins is shown in Fig. 1b as a heat map, demonstrating a distinct pattern of red "hits" for each ototoxin. Upon re-screening and testing of the dose–response matrix, seven compounds were shown to protect hair cells from neomycin damage and six from continuous gentamicin exposure (Fig. 1c; Table 2). Five compounds exhibited at least partial protection from both aminoglycosides: the p53 inhibitor pifithrin-α (PFTα), the Omi/HtrA2 protease inhibitor Ucf-101, the serine protease inhibitor FUT-175, the proteasome inhibitor Z-LLF-CHO, and the autophagy inhibitor 3-MA (Table 2). FUT-175, Z-LLF-CHO, and 3-MA also protected hair cells from cisplatin toxicity. The calpain and cathepsin inhibitor leupeptin significantly protected hair cells from neomycin damage, and to a lesser degree cisplatin damage, but leupeptin was toxic to the animals when combined with continuous gentamicin treatment. D-methionine, an antioxidant, significantly protected hair cells from gentamicin or cisplatin damage but not from neomycin toxicity (Table 2). Bax inhibition protected hair cells from neomycin damage and to a lesser degree from acute gentamicin toxicity (Table 2). p53 inhibition robustly protected hair cells from continuous gentamicin damage, with more limited yet significant protection seen from acute neomycin or acute gentamicin exposure. These "hits" suggest a complex interplay of related pathways underlie hair cell responses to ototoxic damage, with each ototoxin activating a distinct, yet partially overlapping, set of available cell death pathways. Statistics for each compound/ototoxin combination are presented in Table 2.

Inhibitors in the initial library were selected partially because they had known intracellular targets. Nonetheless, it is possible that protection was conferred by a compound blocking ototoxin uptake rather than via inhibition of cell signaling. We assayed uptake using GTTR, a fluorescently conjugated form of gentamicin [55, 56]. While we did not quantify GTTR fluorescence, qualitative assessments were conducted on a minimum of four fish and five neuromasts per fish for each inhibitor, and fluorescent intensity was qualitatively similar across neuromasts and animals. Of the eight inhibitors that demonstrated confirmed protection, only FUT-175 attenuated GTTR entry into hair cells, as shown in Fig. 2.

We focus here on examples of inhibitors with different protection profiles to illustrate the patterns of pathway activation. As shown in Fig. 3, the proteasome inhibitor

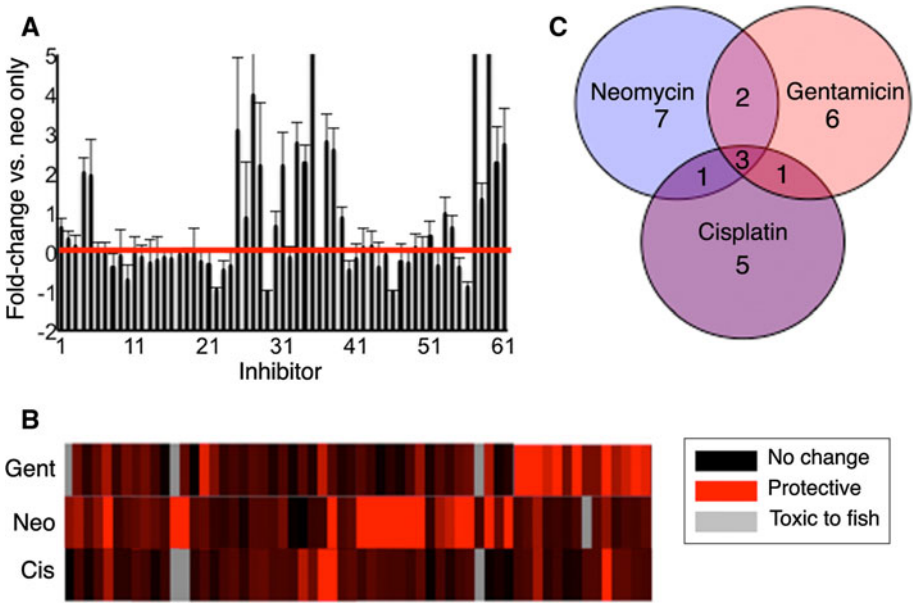

Fig. 1 Screening a cell death inhibitor library for compounds that modulate ototoxin-induced hair cell death in the zebrafish lateral line. **a** Screen results for hair cells treated with inhibitor and neomycin. Hair cell survival is represented as fold-change relative to neomycin only, such that zerofold (*the red line*) denotes the degree of damage caused by neomycin treatment without an inhibitor present. Inhibitors that protected hair cells from neomycin toxicity are visible as bars extending above the red line. Inhibitor identities are given in Table 1. **b** Heat map of all screen data. Ototoxins are represented in *rows*, inhibitors in *columns*. Each *box* denotes a single ototoxin/inhibitor combination. *Black boxes* indicate no protection (no change relative to ototoxin only), *red boxes* are inhibitors that protected hair cells from an ototoxin. *Gray boxes* denote inhibitor/ototoxin combinations that were toxic to the fish. **c** Venn diagram describing the number of inhibitor "hits" that protected hair cells from damage due to each ototoxin. Some inhibitors protected hair cells from damage due to multiple ototoxins, as indicated in the overlapping regions. The *numbers* represent confirmed hits that were verified in triplicate. N = 7–12 animals per treatment, data in (**a**) are presented as mean + 1 SD (Color figure online)

Z-LLF-CHO offers partial protection against all ototoxins tested. 10–50 μM Z-LLF-CHO significantly protected hair cells from 200 μM acute neomycin with 25 μM exhibiting the desired characteristics of optimal protection and minimal toxicity when no ototoxin was present (Fig. 3a and data not shown). Protection was also evident when parvalbumin-labeled hair cells were assessed, demonstrating that DASPEI scores with Z-LLF-CHO reflect hair cell preservation (Fig. 3b). In additional dose–response experiments, 25 μM Z-LLF-CHO robustly protected hair cells from acute neomycin or acute gentamicin toxicity, even at high ototoxin concentrations (Fig. 3c, d). Significant protection from continuous gentamicin was also observed, although this protection was only evident for low concentrations of gentamicin (Fig. 3e). In contrast, modest protection from a relatively high concentration of cisplatin (750 μM) was observed, with no protection seen at lower cisplatin doses (Fig. 3f). Z-LLF-CHO appeared mildly ototoxic during continuous exposure experiments (i.e., Fig. 3e, f, dashed line, 0 ototoxin points).

In contrast to the relatively broad protection offered by Z-LLF-CHO, the antioxidant D-methionine exhibited a more narrow protection profile. As shown in Fig. 4, 5 mM D-methionine significantly protected hair cells from either acute or continuous gentamicin exposure or from cisplatin toxicity. However, this protection was limited, with incomplete hair cell survival seen in all cases. No protection was observed when fish were treated with neomycin in the presence of D-methionine.

The cell death inhibitor library contained multiple caspase inhibitors, including Ac-VAD-CHO and Boc-D-Fmk. No caspase inhibitor manifested as a "hit" during our screen. While we did not follow up on the negative results for the majority of library compounds, given the central importance of caspases in classical PCD we chose to examine putative caspase involvement more thoroughly. We conducted additional experiments with variable concentrations of the pan-caspase inhibitor Z-VAD-Fmk (Z-VAD). As shown in Fig. 5, no concentration of Z-VAD significantly protected hair cells from either aminoglycoside. Previous studies of caspase inhibition by 300 μM Z-VAD report successful prevention of caspase activation and of programmed cell death in zebrafish embryos, including chemical toxicity of neurons and radiation-induced damage [57, 58]. These data suggest that Z-VAD would confer protection if caspase activation were necessary for lateral line hair cell death.

We also found that inhibition of protein synthesis failed to protect lateral line hair cells. As shown in Fig. 6, no concentration of cycloheximide used here protected hair cells from any of our selected ototoxins at any

Table 2 Confirmed hits from the inhibitor screen

Compound name	Molecule or pathway targeted	Maximum effective concentration	2-way ANOVA statistics (numbers in parentheses indicate concentrations that were significantly different in post hoc testing)			
			Acute neo	Acute gent	Continuous gent	Continuous cis
Bax channel blocker	Bax	5 μM	$F_{1,88} = 307.00$, $p < 0.001$ (100, 200, 400 μM)	$F_{1,92} = 13.76$, $p < 0.001$ (200, 400 μM)	$F_{1,89} = 0.65$, $p = 0.42$	T
Pifithrin (PFT)-α	p53	50 μM	$F_{1,88} = 40.86$, $p < 0.001$ (100, 400 μM)	$F_{1,92} = 35.52$, $p < 0.001$ (50, 100 μM)	$F_{1,86} = 563.30$, $p < 0.001$ (50, 100, 200, 400 μM)	$F_{1,56} = 2.06$, $p = 0.16$
Ucf-101	Omi/HtrA2	5 μM	$F_{1,84} = 50.78$, $p < 0.001$ (50, 100 μM)	$F_{1,94} = 27.86$, $p < 0.001$ (100, 200 μM)	$F_{1,89} = 79.13$, $p < 0.001$ (100, 200 μM)	$F_{1,82} = 2.09$, $p = 0.15$
FUT-175	Serine proteases	10 μM	$F_{1,86} = 457.70$, $p < 0.001$ (50, 100, 200, 400 μM)	$F_{1,86} = 449.1$, $p < 0.001$ (50, 100, 200, 400 μM)	$F_{1,88} = 807.00$, $p < 0.001$ (50, 100, 200, 400 μM)	$F_{1,83} = 202.50$, $p < 0.001$ (500, 750, 1000 μM)
Z-LLF-CHO	Proteasome	25 μM	$F_{1,81} = 158.60$, $p < 0.001$ (100, 200, 400 μM)	$F_{1,88} = 150.80$, $p < 0.001$ (50, 100, 200, 400 μM)	$F_{1,86} = 174.4$, $p < 0.001$ (50, 100 μM)	$F_{1,68} = 9.92$, $p = 0.002$ (750 μM)
Leupeptin	Calpains	500 μM	$F_{1,88} = 96.36$, $p < 0.001$ (50, 200, 400 μM)	$F_{1,82} = 1.47$, $p = 0.23$	T	$F_{1,80} = 54.55$, $p < 0.001$ (250 μM)
3-MA	Autophagy	5 mM	$F_{1,88} = 142.40$, $p < 0.001$ (50, 100, 200, μM)	$F_{1,83} = 18.13$, $p < 0.001$ (50, 100 μM)	$F_{1,79} = 7.11$, $p = 0.009$ (posthoc NS)	$F_{1,83} = 20.43$, $p < 0.001$ (250, 750 μM)
D-methionine	Oxidative stress	5 mM	$F_{1,75} = 0.28$, $p = 0.59$	$F_{1,83} = 44.20$, $p < 0.001$ (50, 100, 400 μM)	$F_{1,90} = 129.60$, $p < 0.001$ (50, 100, 200 μM)	$F_{1,61} = 33.89$, $p < 0.001$ (250, 500, 1000 μM)

The maximum effective concentration was determined by testing a range of inhibitor doses with static ototoxin concentrations. The maximum effective concentration was then used in a 2-way dose–response curve with variable ototoxin concentrations. Significant 2-way dose response curves are indicated with significance values below. Only the main effect of the inhibitor is reported here, as the main effect of ototoxin concentration is significant in all experiments. Ototoxin concentrations for which a given inhibitor offered significant protection are given in parentheses (Bonferroni-corrected posthoc analysis). Cases where a given inhibitor did not offer significant protection are in gray. T denotes a combination that was toxic to the fish. Dose–response curves with Z-LLF-CHO and D-Methionine are shown in Figs. 3 and 4, respectively

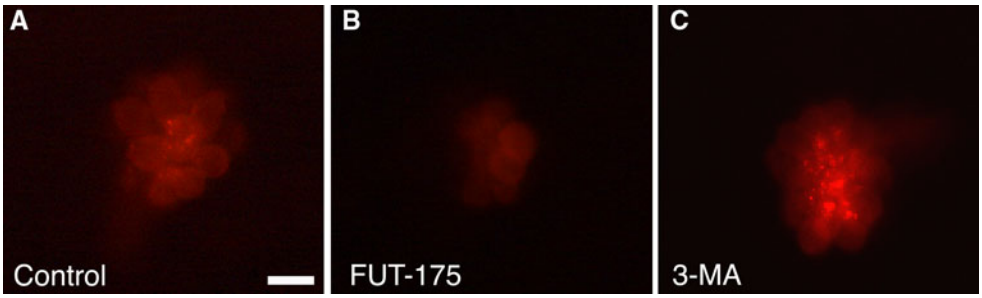

Fig. 2 100 μM GTTR is readily taken up by **a** control hair cells, while **b** 10 μM FUT-175 attenuates GTTR uptake. **c** 5 mM 3-MA does not block GTTR uptake. Scale bar in A is 5 μm and applies to all panels (Color figure online)

concentration. On the other hand, cycloheximide alone (>1 μM) was sufficient to cause hair cell loss (Fig. 6a). Concentrations of 50–350 μM cycloheximide have been previously shown to inhibit protein translation in larval zebrafish [62, 63]. These results are consistent with the interpretation that neither aminoglycoside- nor cisplatin-induced hair cell death in the zebrafish lateral line is dependent on new protein synthesis; it appears to be solely controlled by post-translational cell death mechanisms.

Discussion

The pattern of protection seen in this cell death inhibitor library screen indicates that there is considerable overlap in the cell death pathways activated by the aminoglycoside antibiotics neomycin and gentamicin and the anti-neoplastic agent cisplatin in the zebrafish lateral line. Some inhibitors such as leupeptin have multiple intracellular targets [64, 65], so the precise identification of each cell death molecule is not known in all cases. However, based on the magnitude of protection seen with each inhibitor/ototoxin combination, different pathways appear more important for cell death responses to different ototoxins. For example, the Omi/HtrA2 serine protease inhibitor Ucf-101 protected hair cells from damage due to both aminoglycosides, but robust protection was only evident in combination with continuous gentamicin exposure. On the other hand, the proteasome inhibitor Z-LLF-CHO robustly protected hair cells from acute neomycin or acute gentamicin toxicity, but limited protection was seen using continuous gentamicin or cisplatin. Translation inhibition with cycloheximide and caspase inhibition failed to prevent hair cell death due to any ototoxin examined, suggesting that lateral line hair cell death is not dependent on these processes.

Cell death profiles in ototoxicity

These data suggest that a complex network of interconnected pathways contributes to drug-induced hair cell death in the lateral line system. Prominent among these are protein degradation pathways. Inhibition of the mitochondrial-specific protease Omi/HtrA2 protected hair cells from gentamicin toxicity, and, to a lesser extent neomycin damage. Omi/HtrA2 has been linked to caspase-independent cell death in culture, likely via its serine protease activity [66]. Leupeptin, a calpain and cathepsin inhibitor, significantly protected hair cells from neomycin toxicity and offered slight but significant protection against cisplatin damage. Aminoglycoside treatment increases calpain activity in vivo and leupeptin has been previously shown to protect hair cells from gentamicin toxicity in mammalian inner ear explants [67–69], although no protection from gentamicin was noted in the present in vivo study due to the toxicity of continuous exposure to leupeptin and gentamicin. Bcl2 proteins and p53 are both reported calpain substrates. Inhibition of the Bcl2 family member Bax protected hair cells from neomycin damage, suggesting that calpains and Bax could potentially interact in neomycin-treated hair cells. p53 inhibition conferred protection from both aminoglycosides, and p53 can interact both transcriptionally and post-translationally with several Bcl2 proteins, including Bax [70–72]. Bcl2 proteins and p53 have previously been implicated in ototoxicity, although specific roles for Bax and p53 in aminoglycoside-induced hair cell death have not been reported [73–75]. The role of Bcl2 proteins and p53 in aminoglycoside ototoxicity is an area of ongoing research in our group.

In addition to targeted protein cleavage by specific proteases, the ubiquitin–proteasome system coordinates both ongoing protein degradation in healthy cells and during cell death processes [76]. The proteasome inhibitor Z-LLF-CHO offered robust protection from neomycin and gentamicin toxicity, and slight protection from cisplatin exposure. Proteasome inhibition is generally cytotoxic and targeted proteasome inhibitors are under consideration as chemotherapy drugs [76–78]. However, proteasome function promotes cell death in sympathetic neurons and the proteasome may activate cell death pathways in some cancer cells by degrading pro-survival Bcl2 family

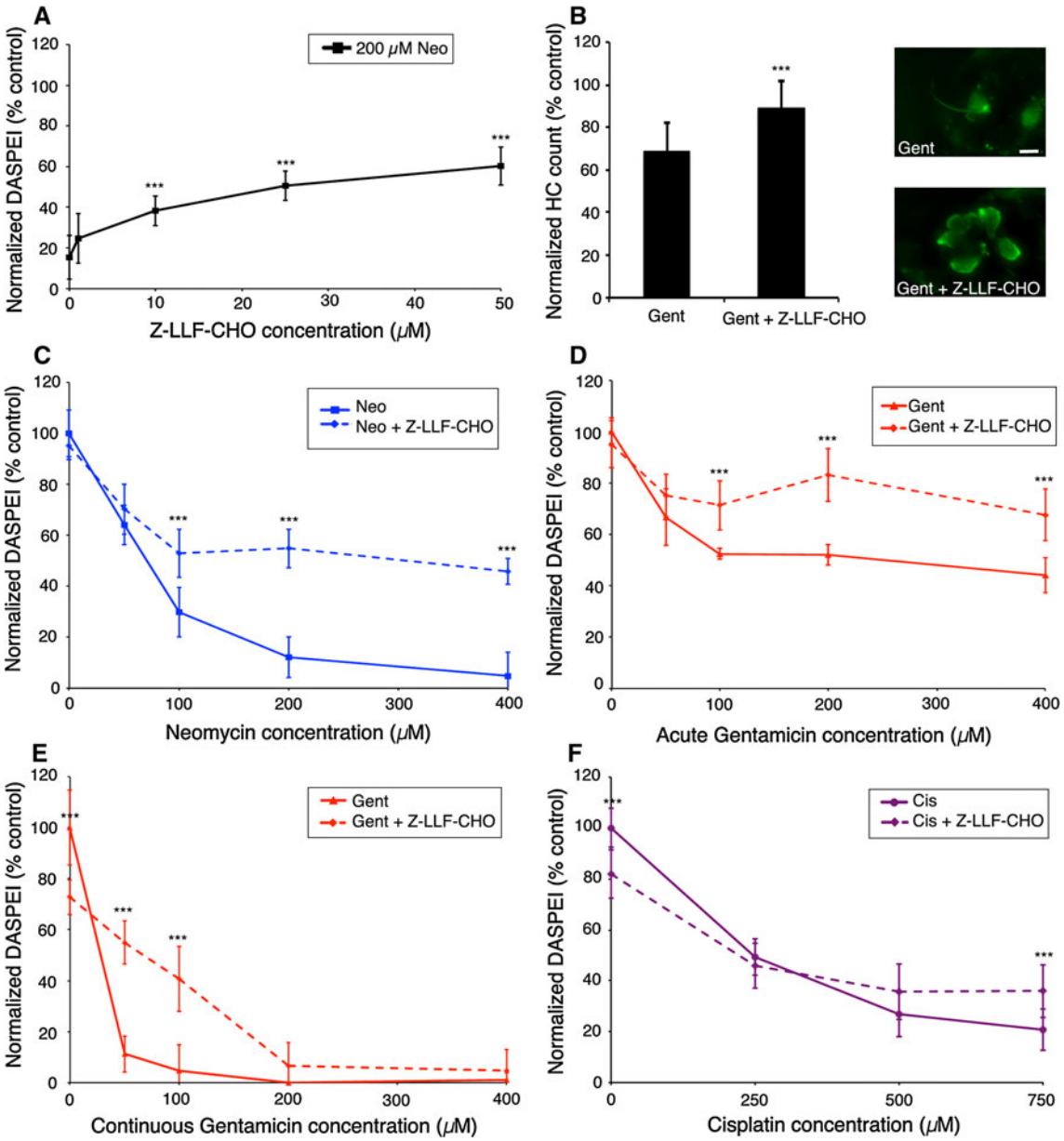

Fig. 3 The proteasome inhibitor Z-LLF-CHO protects hair cells from ototoxin exposure. **a** Z-LLF-CHO provides dose-dependent protection from 200 μM acute neomycin (1-way ANOVA, $F_{4,48} = 37.59$, $p < 0.001$). 25 μM Z-LLF-CHO offered optimal protection without any ototoxicity, and there was not a significant difference in protection between 25 and 50 μM Z-LLF-CHO. **b** Direct counts of parvalbumin-labeled hair cells confirm that Z-LLF-CHO treatment protects hair cells from gentamicin toxicity (*t* test, $p < 0.001$). Fish were treated with 100 μM continuous gentamicin with or without 25 μM Z-LLF-CHO. Images in **b** show examples of labeled hair cells, *scale bar* = 5 μm and applies to *both panels*. **c**–**f** 25 μM Z-LLF-CHO robustly protects hair cells from **c** acute neomycin, **d** acute gentamicin, **e** continuous gentamicin, and **f** continuous cisplatin. Statistics for the dose–response analyses shown in (**c**–**f**) are given in Table 2. Significance values for individual comparisons in Bonferroni-corrected posthoc tests are indicated on the figures, where ***$p < 0.001$. Data are presented as mean ± 1 SD (Color figure online)

members, suggesting that proteasome inhibition may promote cell survival in certain contexts [79–81].

Reactive oxygen species (ROS) production is correlated with aminoglycoside and cisplatin ototoxicity and antioxidant therapies reduce hair cell death and the associated hearing loss in animal models [82–86]. In the present study, D-methionine offered slight but significant protection from gentamicin or cisplatin damage but not from neomycin toxicity. Ton and Parng [87] also noted a protective effect of D-methionine on cisplatin-treated zebrafish hair cells, as well as protection offered by other antioxidants. Despite these findings, it is unclear whether ROS generation provides the main avenue for ototoxicity, is indicative of cell signaling due to altered metabolism, or is a

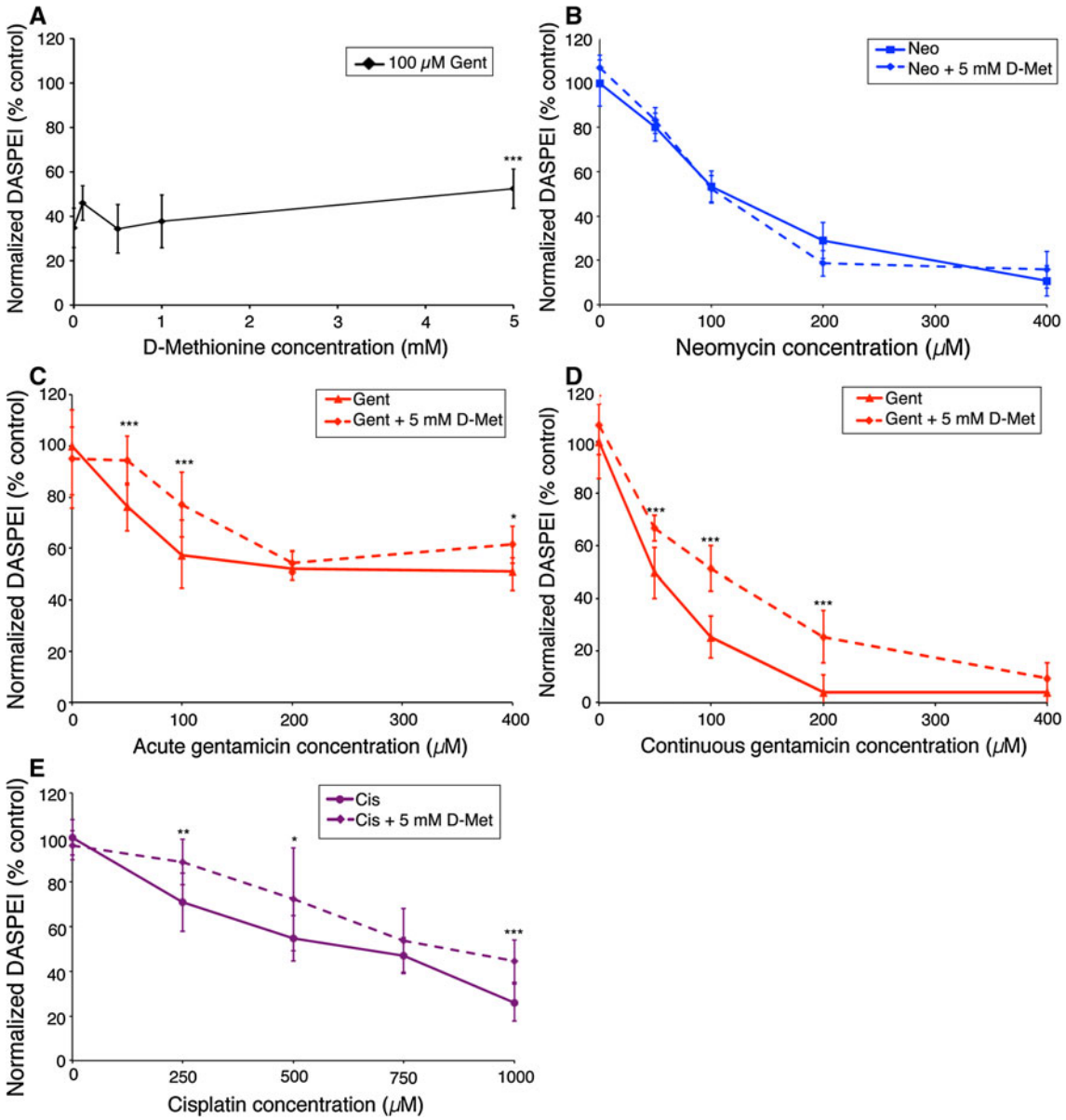

Fig. 4 **a** D-methionine significantly protects hair cells from continuous gentamicin damage (1-way ANOVA, $F_{4,55} = 7.74$, $p < 0.001$), with 5 mM D-met providing optimal protection without overt toxicity. **b** 5 mM D-met does not protect hair cells from neomycin toxicity, while significant protection is offered across much of the dose–response function for acute **c** and continuous **d** gentamicin. Slight but significant protection is also seen from continuous cisplatin exposure **e**. 2-way ANOVA statistics are given in Table 2, significance values from Bonferroni-corrected posthoc analysis are indicated on the figure; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. Data are presented as mean ± 1 SD (Color figure online)

side-effect of mitochondrial or other organelle damage [88–90]. It is also unclear why D-methionine was not protective against neomycin damage. Most aminoglycoside antioxidant studies have employed gentamicin as the damaging agent of choice, although a few studies report that neomycin can also cause ROS formation [91–95]. The full effect of antioxidant therapy as a treatment for neomycin toxicity is therefore unknown, and our results suggest that D-methionine is not sufficient to protect zebrafish lateral line hair cells from neomycin. Other antioxidants such as lipoic acid or salicylate may be more effective [96]. Conversely, neomycin may activate such a large number of cell death pathways in parallel in the lateral line that antioxidant treatment alone is insufficient to prevent hair cell loss. Additional studies are needed to test amongst these competing hypotheses.

Both the autophagy inhibitor 3-MA and the general serine protease inhibitor FUT-175 protected hair cells from all ototoxins surveyed here. Autophagy can promote either cell survival or cell death depending on the context and

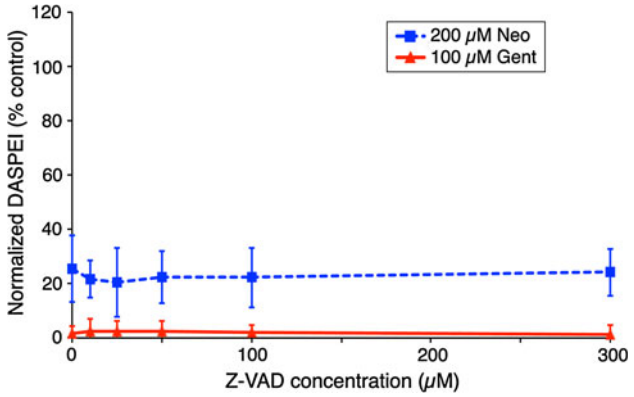

Fig. 5 The general caspase inhibitor Z-VAD does not significantly protect hair cells from 200 μM acute neomycin (1-way ANOVA, $F_{5,52} = 0.30$, $p = 0.91$) or 100 μM continuous gentamicin (1-way ANOVA, $F_{5,56} = 0.20$, $p = 0.96$). Data are presented as mean ± 1 SD (Color figure online)

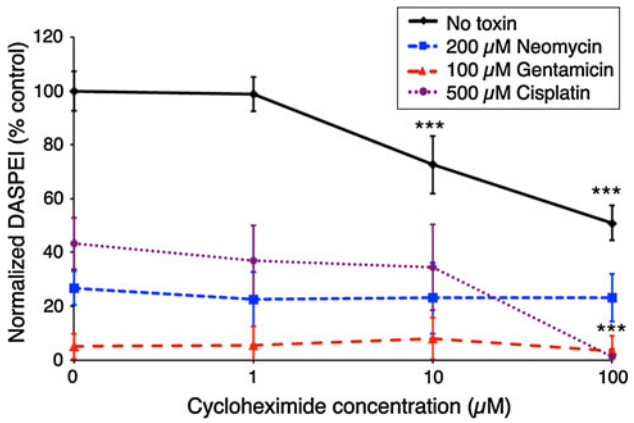

Fig. 6 The protein synthesis inhibitor cycloheximide does not protect hair cells from ototoxic damage. Significant hair cell toxicity is evident following cycloheximide treatment alone (1-way ANOVA, $F_{3,42} = 104.7$, $p < 0.001$), and cycloheximide increased hair cell loss due to cisplatin toxicity (1-way ANOVA, $F_{3,31} = 14.0$, $p < 0.001$). Cycloheximide treatment did not influence acute neomycin-induced hair cell death (1-way ANOVA, $F_{3,40} = 0.40$, $p = 0.75$) nor hair cell death due to continuous gentamicin exposure (1-way ANOVA, $F_{3,43} = 1.11$, $p = 0.35$). *Asterisks* indicate significant pairwise differences using Bonferroni-corrected posthoc testing ($*p < 0.05$, $***p < 0.001$). Data are presented as mean ± SD (Color figure online)

autophagic pathways intersect with classical mitochondrial cell death machinery [97–99]. Pro-survival Bcl2 proteins can inhibit autophagic cell death by associating with Beclin, an important regulator of autophagy [99, 100]. FUT-175 is of particular interest due to its broad protective capacity and clinical use as a complement inhibitor, anti-inflammatory, and anti-coagulant [101–103]. The intracellular targets of FUT-175 are not well understood and the mechanism by which this compound protects hair cells is an area for future investigation. FUT-175 reduced uptake of fluorescently-tagged gentamicin, suggesting that protection may result from blocking ototoxin uptake rather than from intracellular activity. Hair cell entry of aminoglycosides and cisplatin is dependent on active mechanotransduction, so FUT-175 may function as a transduction blocker [55, 104–107]. Alternatively, FUT-175 may protect hair cells by a combination of extracellular and intracellular mechanisms. One potential FUT-175 target is high mobility group box 1 (HMGB1) protein, which is associated with inflammation [108]. HMGB1 can interact with p53 to regulate both autophagy and cell death, suggesting a possible connection between FUT-175, 3-MA, p53 and Bcl2 proteins [109].

How do hair cells die?

Collectively our data are consistent with caspase-independent cell death (CICD) via intrinsic mitochondrial pathway(s). Like apoptosis, CICD is dependent on activation of pro-cell death Bcl2 family proteins such as Bax and p53 mitochondrial activity [13, 110]. Calpains are reported to act upstream of Bax activation in CICD and the calpain inhibitor leupeptin protects hair cells from aminoglycoside-induced hair cell death [13, 67, 69, present study]. Inhibition of calpains, Bax, or p53 protected hair cells from neomycin damage in the present study, suggesting that neomycin may activate this CICD pathway. In contrast, gentamicin-induced hair cell death in the zebrafish lateral line appears to require p53 and the mitochondrial protein Omi/HtrA2 but not Bax. Collectively, these results implicate intrinsic mitochondrial-associated cell death pathways, consistent with prior studies [17, 25, 75]. One central player in many caspase-independent cell death pathways is apoptosis-inducing factor (AIF), and Bax activity may promote AIF translocation from the mitochondria to the nucleus [12, 13, 111]. AIF can also induce Bax-independent cell death in cultured neurons, suggesting that mitochondrial release of AIF does not absolutely require Bax [112]. As there is no established pharmacologic inhibitor of AIF we did not test its function in our current study, but consider it a prime target of interest for future research.

The possible requirement for caspases in hair cell death is still unresolved. Previous research suggests that caspase activation is necessary for aminoglycoside-induced hair cell death both in vitro in mammalian inner ear cultures and in vivo in chick [24, 26, 27, 113, 114]. Williams and Holder showed that Z-VAD reduced hair cell death in the zebrafish lateral line caused by neomycin treatment [115]. Williams and Holder employed a much lower concentration of neomycin than was used in the present study (10 μM, vs. 50–400 μM used here) and they performed their experiment in low-calcium conditions, which facilitates aminoglycoside uptake by hair cells [51, 105, 115]. However, pharmacologic caspase inhibition did not protect

hair cells from aminoglycoside damage in the present study. It is therefore possible that differences in calcium concentrations or other treatment conditions led to activation of different cell death pathways. Experimental discrepancies, such as differences in concentrations or dosing schedules, could similarly account for the variable caspase dependence or independence reported in other aminoglycoside-treated vertebrate ears [15, 27, 113]. Consistent with this hypothesis are reports that caspase inhibition protects hair cells from a limited dose of neomycin in vitro but that protection is lost at higher concentrations [69]. Moreover, some studies report morphological changes consistent with multiple cell death mechanisms within a single aminoglycoside-treated epithelium, suggesting activation of classical apoptotic pathways as well as alternative death pathways [15, 116, 117]. It is possible that caspase-dependent cell death accounts for a small fraction of the total cell death observed in our study and that more sensitive assessment methods are necessary to detect this contribution.

It is important to note that the cell death profiling approach used here has several caveats. Given the nature of the screening process, false negatives are likely, as we screened a single inhibitor concentration rather than a range of doses. Given the large number of initial "hits" in our screen with neomycin (approximately 1/3 of the library, Fig. 1a), we think that our inhibitor concentration choices were within the biologically active range for these compounds. Compounds reported to be cell-permeable by the manufacturer were selected whenever possible but it is feasible that some compounds did not enter hair cells and therefore could not access the appropriate intracellular targets. In addition, while the library components were selected to encompass a range of characterized cell death-associated molecules, at 61 compounds this library is small and potentially important death pathway molecules were not included, such as compounds targeting apoptosis inducing factor (AIF) or inhibitor of apoptosis (IAP). It is therefore likely that we missed several molecular steps in the cell death cascade initiated by each ototoxin. The inhibitor library was carefully selected to include compounds with well-characterized molecular targets. Still, off-target interactions are possible, as are physical interactions between inhibitor and ototoxin. Future experiments will use a combination of pharmacology and genetic manipulation to more thoroughly validate the pathway profiles generated in the present study and to target the "missing links" in these profiles. Additionally, as this screen employed an inhibitor approach, complementary screens using cell death activation assays would offer additional validation and target pathways not identified in our study. Finally, additional experiments are required to compare the pathways identified in the zebrafish lateral line with those activated in the mammalian inner ear.

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