Received: 4 July 2017 | Revised: 29 August 2017 | Accepted: 10 September 2017

DOI: 10.1002/jat.3547

WILEY Journal of Applied Toxicology

RESEARCH ARTICLE

A mitochondrial targeting tetrapeptide Bendavia protects lateral line hair cells from gentamicin exposure

Xiao Kuang | Yanhui Sun | Zhenjie Wang | Shuang Zhou | Hongzhuo Liu

School of Pharmacy, Shenyang Pharmaceutical University, Shenyang 110016, People's Republic of China

Correspondence
Hongzhuo Liu, School of Pharmacy, Shenyang Pharmaceutical University, No. 103 Wenhua Road, Shenyang 110016, People's Republic of China
Email: liuhongzhuo@syphu.edu.cn

Funding information
National Natural Science Foundation of China, Grant/Award Number: 81473162

Abstract

The hearing loss induced by aminoglycosides is caused by the permanent loss of mechanosensory hair cells of the inner ear. The aim of the present study is therefore to evaluate the protective effect of Bendavia, a novel antioxidant, on gentamicin-induced hair cell damage in zebrafish lateral lines. The results demonstrated the pretreatment of Bendavia exhibited dose-dependent protection against gentamicin in both acute and chronic exposure. We found that Bendavia at 150 μM conferred optimal protection from either acute or chronic exposure with ototoxin. Bendavia reduced uptake of fluorescent-tagged gentamicin via mechanoelectrical transduction channels, suggesting its protective effects may be partially due to decreasing ototoxic molecule uptake. The intracellular death pathways inhibition triggered by gentamicin might be also included as no blockage of gentamicin was observed. Our data suggest that Bendavia represents a novel otoprotective drug that might provide a therapeutic alternative for patients receiving aminoglycoside treatment.

KEYWORDS

aminoglycosides, Bendavia, gentamicin, hearing loss, ototoxicity, zebrafish lateral lines

1 | INTRODUCTION

Aminoglycosides are broad-spectrum antibiotics that are particularly useful in being active against aerobic, gram-negative bacteria. Despite their serious side effects, such as irreversible hearing loss, the drugs are commonly prescribed in some situations, including prevention and treatment of life-threatening sepsis in newborns or immunocompromised persons (Courjon et al., 2017; Johnson & Messier, 2016; Mishra et al., 1998). In addition, they are used as part of the regimen against tuberculosis due to their low cost in developing countries.

As aminoglycosides are known to induce hearing loss, numerous studies have been conducted to understand better the cellular mechanisms underlying drug-induced inner ear damage. A lot of evidence suggested that oxidative stress-induced apoptosis and necrosis in the hair cells of the cochlea, marginal cells and stria vascularis (Lanvers-Kaminsky, Zehnhoff-Dinnesen, Parfitt, & Ciarimboli, 2017). Once loaded into hair cells, aminoglycosides incline to bind to mitochondria, which formed bacterial ribosome-like structures (Hutchin & Cortopassi, 1994; Lynch & Puglisi, 2001). Previous studies demonstrated that aminoglycosides are capable of direct interaction with mitochondrial translation machinery, altering the mitochondrial proton gradient and then leading to the collapse of the mitochondrial membrane potential. Moreover, aminoglycosides promote reactive oxygen species (ROS) formation and then induce apoptotic-like cell death via inhibition of mitochondrial protein biosynthesis. Owing to the key role of ROS in aminoglycoside-induced ototoxicity, a number of antioxidants or scavengers of ROS have been currently used in clinical trials to attenuate ototoxicity (Kingwell, 2016). However, as none of treatments was approved for the treatments of drug-induced ototoxicity, there remain imperious demands to identify novel protective drugs.

Bendavia (or SS-31) is a mitochondrial targeting tetrapeptide that could scavenge mitochondrial ROS by inhibition of mitochondrial permeability transition pore and stabilization of cardiolipin (Szeto & Birk, 2014). It represents the first of a class of new chemical entities that selectively target mitochondrial cardiolipin to improve mitochondrial plasticity and restore optimal bioenergetics (Shi et al., 2015; Szeto, 2014). Because of the novel mechanism of action, Bendavia might provide therapeutic benefits in mitochondrial disease, including cardiovascular, renal, ophthalmic, metabolic and skeletal muscle disorders (Szeto & Birk, 2014). As the preclinical effectiveness of Bendavia for reducing myocardial ischemia–reperfusion injury and

its safety, tolerability and pharmacokinetics in healthy subjects, it has been pushed in a clinical Phase II trial that focused in ischemia–reperfusion injury and microvascular injuries in patients experiencing acute ST-segment elevation myocardial infarction (Daaboul et al., 2016). Another second Phase II trial involved Bendavia is on the way for the treatment of acute kidney injury (Kezic, Spasojevic, Lezaic, & Bajcetic, 2016) and renal microvascular dysfunction in hypertension (Liu, Soong, Seshan, & Szeto, 2014). In addition, it has also been scheduled to evaluate potential efficacy in the treatment of heart failure and diabetic macular edema clinically (Szeto & Birk, 2014).

The aim of the present study was to evaluate the possible protecting role of Bendavia on gentamicin-induced hair cell loss using zebrafish as an experimental animal model and to investigate the possible underlying mechanisms.

2 | MATERIALS AND METHODS

2.1 | Drugs and chemicals

D-Arg-2′6′-dimethylTyr-Lys-Phe-NH_2 (Bendavia) was purchased from GL Biochem Ltd. (Shanghai, China) and gentamicin sulfate (GT) was purchased from Dalian Meilun Biotech Co., Ltd. (Dalian, China). 2-[4-(dimethylamino)styryl]-1-ethylpyridinium iodide (DASPEI) and MS-222 were obtained from Sigma-Aldrich (St. Louis, MO, USA). Texas Red (TR) succinimidyl esters and FM1-43FX were both purchased from Molecular Probes, Inc. (Invitrogen, Eugene, OR, USA).

2.2 | Animals

Zebrafish embryos were obtained by paired mating of wild-type adult fish (China Zebrafish Resource Center, Wuhan, China) and directly purchased from Ezerinka Biotechnology Co., Ltd. (Nanjing, China). Embryos were raised at 28.5°C in embryo medium (EM) consisting 5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$ and 0.33 mM $MgSO_4$ at a density of 50 animals per 100 mm^2 Petri dish. All zebrafish protocols were approved by the Shenyang Pharmaceutical University Institutional Animal Care and Use Committee. All methods were performed in accordance with the guidelines of the Animal Care Ethics Committee of Shenyang Pharmaceutical University Medical Center and National Institutes of Health (NIH) guidelines.

2.3 | Gentamicin-induced hair cell damage

Stock solution of gentamicin was diluted in EM to final concentrations of 0–800 μM for evaluating dose–response testing for gentamicin in zebrafish lateral line hair cells. Larvae (5 days postfertilization [dpf]) were incubated with ototoxic agent for 1 hour (termed "acute exposure") or 6 hours (termed "continuous exposure") in a range of concentrations of gentamicin as it induced hair cell damage with differing mechanisms (Owens et al., 2009). The exposed larvae were then rinsed 4× with fresh EM. The treated larvae in the acute exposure were allowed to recover for 1 hour before hair cell assessment. On the contrary, the larvae in the continuous exposure were examined immediately after the exposure period.

2.4 | Toxicity assessment of Bendavia in hair cells

Although Bendavia was regarded as safe and good tolerable in human via i.v. or p.o. administration, no reports showed its impact on hair cells. For this purpose, larvae (5 dpf) were exposed to Bendavia at 15, 150, 300 and 1500 μM concentrations for 1 or 6 hours (exposure duration matching duration used for gentamicin-induced damage) and then the potential toxicity of Bendavia was identified with DASPEI score.

2.5 | Dose–response analysis of Bendavia in gentamicin-induced hair cell damage

To assess the protective effect of Bendavia, the compound was tested by dose–response curve. Briefly, larvae (n = 10 per group) were incubated for 1 hour with Bendavia at the concentrations of 1.5, 15, 75, 150 and 225 μM. Then the pretreated larvae were further co-incubated with gentamicin for another 1 or 6 hours. The EM- and gentamicin-treated groups were used as the negative and positive control respectively.

2.6 | Hair cell assessment

For rapid *in vivo* assessment, zebrafish lateral line hair cells were labeled with the mitochondrial dye DASPEI, which is taken up by living cells as a function of mitochondrial membrane potential (MMP) (Coffin, Williamson, Mamiya, Raible, & Rubel, 2013). The post-treated larvae were incubated in the EM containing 0.005% DASPEI for 20 minutes before anesthetization with 0.02% of MS-222 (Harris et al., 2003). Then the larvae were rinsed 3× with fresh EM. DASPEI labeling was evaluated on a Leica epifluorescent microscope (Chroma Technologies, Brattleboro, VT, USA) for 10 neuromasts (SO1, SO2, SO3, O1, O2, MI1 and P1–4). Each neuromast was assigned a score of 0 (no/little staining), 1 (reduced staining) or 2 (wild-type-like staining) for a composite score of 0–20. DASPEI scores were averaged for each group and normalized as a percentage of mock-treated controls.

As the DASPEI assessment is semi-quantitative, the immunocytochemistry was used to validate the protection of Bendavia against gentamicin-induced hair cell damage by direct hair cell counts. Briefly, the post-treated larvae were killed with an overdose of MS-222 and fixed with 4% paraformaldehyde overnight at 4°C. Then the larvae were rinsed in phosphate-buffered saline (PBS) and incubated in blocking solution (1% Triton-X, 5% normal goat serum [NGS] in PBS) for 1–2 hours at room temperature. To label the hair cells, the larvae were incubated overnight at 4°C in parvalbumin primary antibody (monoclonal, 1:500 in 1% Triton-X, 1% NGS, in PBS; GenTex, San Antonio, TX, USA), then rinsed in 1% Triton-X in PBS (PBS-T) and transferred to Alexa 488 goat antirabbit fluorescent secondary antibody solution (1:500 in 1% Triton-X, 1% NGS, in PBS; Invitrogen, Eugene, OR, USA) for a 2–4 hour incubation at room temperature. Following two to three additional rinses in PBS-T, the larvae were stored in 1:1 PBS/glycerol at 4°C before assessment on a Leica epifluorescent microscope. The hair cell number was quantified in four neuromasts (O1, O2, MI1, M2 and OP1) per larvae, summed to calculate one value per animal and averaged for each group. Results

were compared as the mean hair cell survival as a percentage of the group treated only in EM (Thomas et al., 2015).

2.7 | Gentamicin uptake assay

To evaluate the impact of Bendavia on gentamicin uptake by the hair cells, the larvae were incubated for 1 hour in the optimal concentration of Bendavia, followed by 5 or 20 minutes co-incubation with gentamicin tagged with fluorophore TR (GTTR, 100 μM) (Ou et al., 2012; Wang & Steyger, 2009). Excess fluorophore was removed with two to three rinses in fresh EM and the larvae were observed under a confocal laser scanning fluorescent microscopy (Leica, Heidelberg, Germany). Neuromast GTTR intensity was assessed qualitatively by Image-Pro Plus 6.0 (Media Cybernetics Inc., Rockville, MD, USA).

2.8 | FM1-43FX uptake test

To assess the transduction channel integrity, larvae (5 dpf) were exposed to Bendavia for 1 hour and then 1 μM of FM1-43FX were added for the treatment for 45 seconds. Followed by 3× rinses with fresh EM, neuromasts were then observed using Leica epifluorescent microscope at 3 minutes after addition of FM1-43FX to assess uptake (Kruger et al., 2016).

2.9 | Statistical analysis

We used Origin 8.0 (OriginLab Inc., Northampton, MA, USA) for the statistical analysis. All results were represented as mean ± SD. Hair cell counts were evaluated by *t*-test and one-way analysis of variance was used for multiple comparisons. Significance was accepted for $P < 0.05$.

3 | RESULTS

3.1 | Dose–response for gentamicin to hair cells

Earlier studies have demonstrated that gentamicin-induced both acute and slow mechanisms that are distinct from one another. We first conducted the dose–response of gentamicin studies in acute or chronic exposures. Figure 1 illustrated that the dose–response relationship by analyzing DASPEI labeling of neuromasts after acute or chronic exposure to gentamicin ranging from 0 to 800 μM. As shown in Figure 1(A), DASPEI scores were 55.6 ± 14.2% of the control group scores following 200 μM of gentamicin, which reflected significant hair cell loss after acute exposure. Hair cell survival dropped to 45.9 ± 13.6% and 39.0 ± 23.3% of the control group score following 400 and 800 μM of gentamicin exposure, respectively. On the contrary, chronic gentamicin exposure led to significant hair cell loss beyond that observed in the acute exposure. Figure 1B, showed that the DASPEI scores were 44.1 ± 17.2% of the control following only 2 μM of gentamicin, while it sharply declined to 14.0 ± 14.5% following 5 μM of gentamicin and nearly 0% following 10 μM of gentamicin.

3.2 | Potential toxicity response of Bendavia on hair cells

Concerning the possible toxicity of Bendavia itself, the survival of hair cells upon the exposure of Bendavia were first evaluated in this investigation. As shown in Figure 2A, Bendavia at 300 μM itself seemed to be harmful to hair cells compared with the negative control ($P < 0.01$) after acute exposure. On the other hand, it was observed that Bendavia at 150 μM caused significant, albeit slight, damage to hair cells, with greater damage at 300 μM after chronic exposure (Figure 2B). Our findings of toxicity-related Bendavia were inconsistent with the previous studies, which demonstrated Bendavia to be nontoxic to heart or neuronal cells at dosages ranging 1 nM–1 mM (Zhao, Luo, Giannelli, & Szeto, 2005). It indicated that hair cells might be more susceptible to chemical agents than the other cell lines.

3.3 | Protective effect of Bendavia on gentamicin-induced hair cell damage

Treatment with Bendavia conferred protection from gentamicin in a dose-dependent manner (Figure 3). Pretreatment for 1 hour with 150 μM Bendavia followed by 1 hour of co-treatment with 200 μM GT significantly increased hair cell survival from 55.6 ± 14.2% (GT only) to 77.3 ± 9.7% (Figure 3A; $P < 0.001$). While the same pretreatment

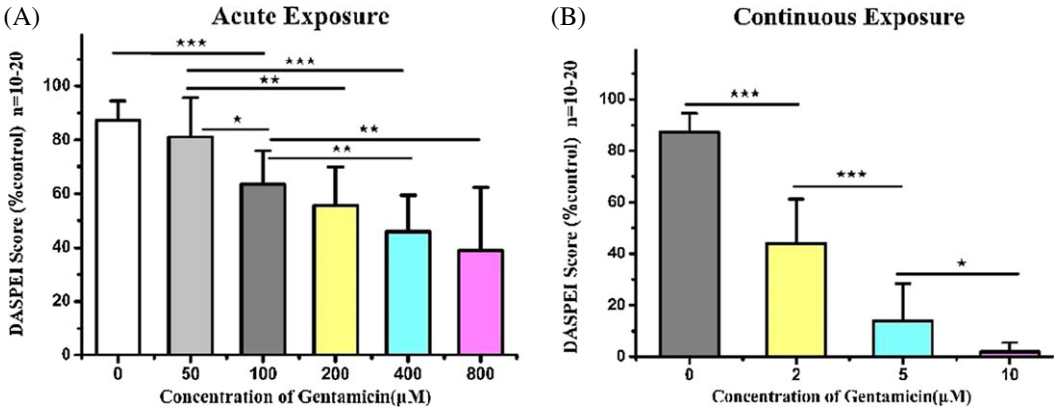

FIGURE 1 Dose–response relationship between neuromast hair cell survival and gentamicin concentration. Zebrafish larvae (5 dpf) was incubated with 200 μM gentamicin for 1 hour (acute exposure) (A), and 2 μM gentamicin for 6 hours (continuous exposure) (B), respectively then immersed in 0.005% DASPEI for 20 minutes (one-way ANOVA, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$) [Colour figure can be viewed at wileyonlinelibrary.com]

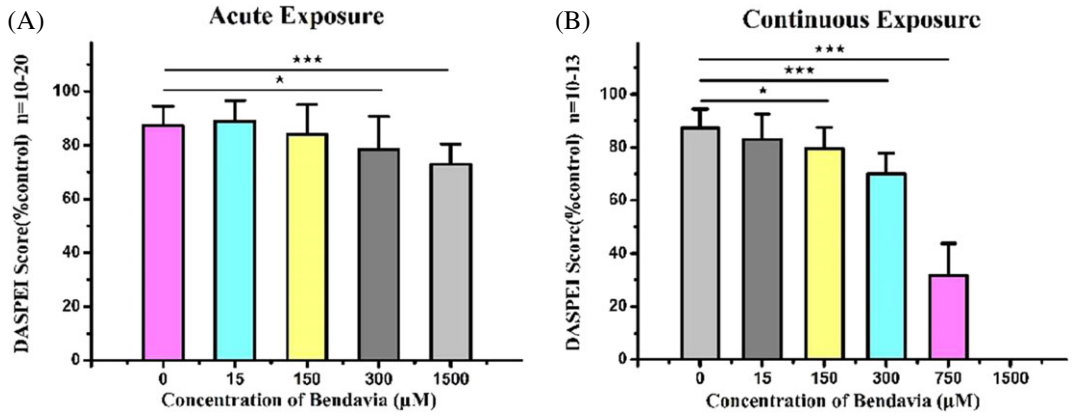

FIGURE 2 Dose–response relationship between neuromast hair cell survival and Bendavia concentrations. Zebrafish larvae (5 dpf) was incubated with Bendavia of various concentrations for 1 hour (acute exposure) (A), and 6 hours (continuous exposure) (B), respectively then immersed in 0.005% DASPEI for 20 minutes. Statistic histogram revealed the conclusion (one-way ANOVA, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$) [Colour figure can be viewed at wileyonlinelibrary.com]

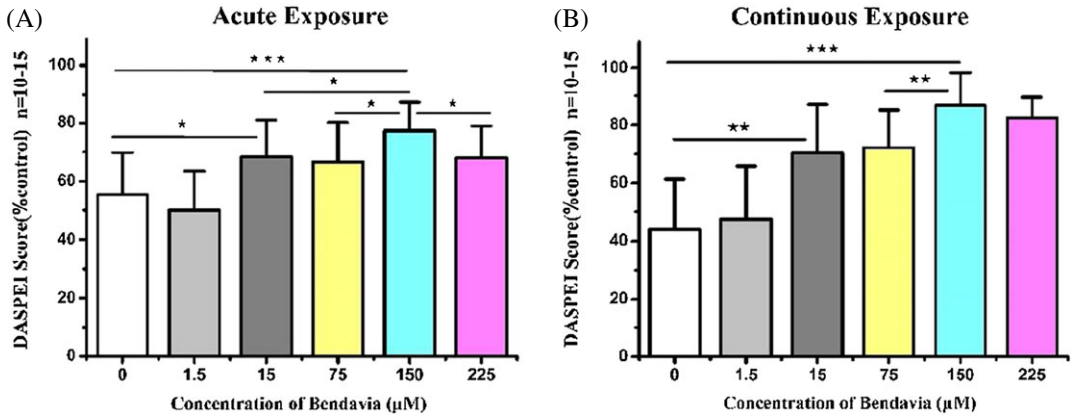

FIGURE 3 Protective effect of different concentrations Bendavia against gentamicin-induced hair cell loss under acute exposure (200 μM gentamicin treated for 1 hour) (A), or chronic exposure (2 μM gentamicin treated for 6 hours) (B), which is indicated by DASPEI staining as a DASPEI score percentage of mock-treated controls in 5 dpf zebrafish larvae (one-way ANOVA, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$) [Colour figure can be viewed at wileyonlinelibrary.com]

with Bendavia followed by co-treatment with 2 μM GT for 6 hours significantly increased hair cell survival from 44.1 ± 17.2% (GT only) to 86.8 ± 11.7% (Figure 3A; $P < 0.001$). Accordingly, Bendavia at 150 μM represented the maximum protection observed since the increase in hair cell survival between doses of 75 μM (66.5 ± 13.6% in acute exposure and 72.1 ± 13.0% in chronic exposure) and 150 μM was significant. Decreased hair cell survival was observed at higher concentrations of Bendavia (225 μM) when compared with that of 150 μM.

We then evaluated whether the protection by a fixed dose of Bendavia would be maintained at higher doses of gentamicin. We found that 150 μM Bendavia resulted in significant protection against 400 μM gentamicin ($P < 0.01$) in acute exposure (Figure 4A) although no significant effect was observed against 100 μM gentamicin. Fortunately, in the presence of 5 or 10 μM gentamicin in chronic exposure, the survival hair cell was still significantly higher, 59.1 ± 13.4% and 62.9 ± 15.1% of DAPEI score respectively, compared to 14.0 ± 14.5% and 2.0 ± 3.5% with gentamicin alone (Figure 4B, $P<0.001$).

To verify DASPEI scores, zebrafish from groups with the same treatment were fixed and hair cells were visualized by means of immunohistochemistry. Direct hair cell counts were then obtained and the results are shown in Figure 5. The data further supported that Bendavia at 150 μM robustly protected hair cells from gentamicin damage as hair cells survived from 31.6 ± 16.7% by gentamicin alone to 86.2 ± 9.2% by the pretreatment of Bendavia in the acute exposure. Concerning the chronic exposure, the survival rate of hair cells upon the pretreatment of Bendavia significantly increased while bare of hair cells remained upon GT alone (from 0% to 79.2%).

3.4 | Bendavia reduced gentamicin uptake into hair cells

To verify gentamicin loading into hair cells, we exposed zebrafish to 100 μM GTTR for 5 or 20 minutes, washed out the zebrafish and then immediately imaged the neuromasts. Bendavia treatment resulted in a significant reduction of GTTR fluorescence to 75.8 ± 28.9% (5 minutes) and 80.6 ± 6.95 (20 minutes) of controls ($P < 0.05$ and $P < 0.001$ respectively; Figure 6). To assess the relationship between MET activity and loading of gentamicin, we also used the fluorescent vital dye, FM 1-43FX, whose entry through MET channels depends on their open probability. Application of 150 μM Bendavia significantly reduced

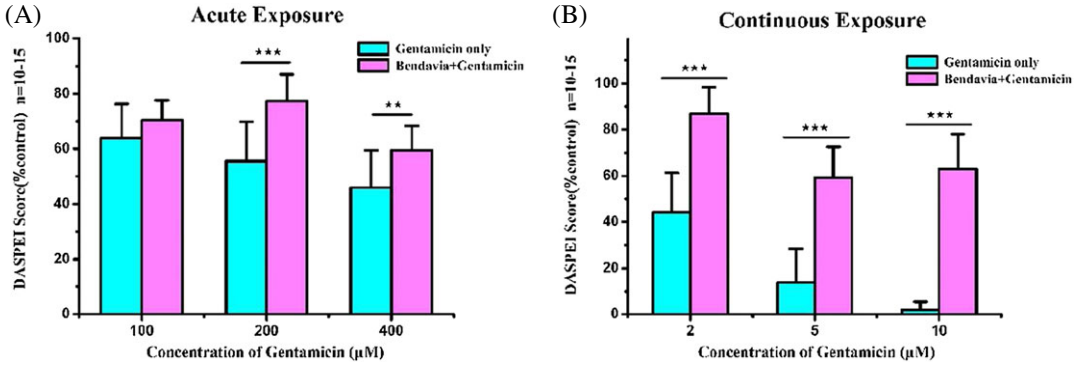

FIGURE 4 Protection by a fixed dose (150 μM) of Bendavia against gentamicin exposure. 150 μM Bendavia was chosen as optimal concentration to conduct dose–response analysis of Bendavia in gentamicin of different concentrations (100, 200 and 400 μM for acute exposure (A); 2, 5 and 10 μM for continuous exposure (B)) induced hair cell damage in 5 dpf zebrafish larvae. Statistic histogram revealed the conclusion (one-way ANOVA, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$) [Colour figure can be viewed at wileyonlinelibrary.com]

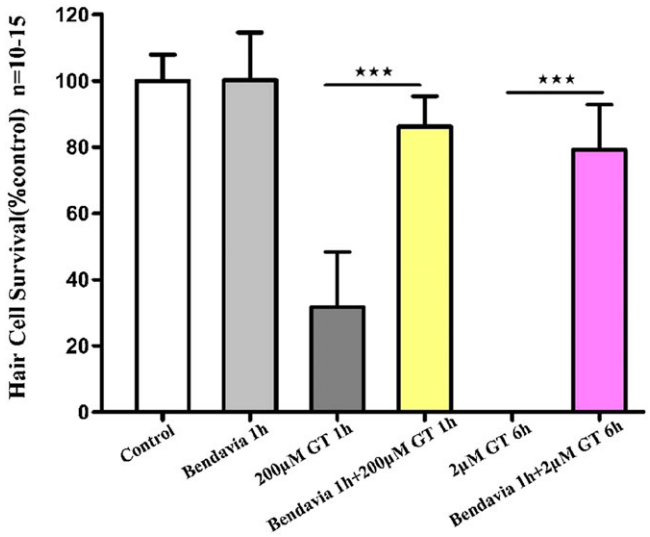

FIGURE 5 Quantitative assay for hair cell survival. Hair cell number was quantified in five neuromasts (O1, O2, MI1, M2 and OP1) per larvae, summed to calculate one value per animal and then averaged for each group. Results were compared as the mean hair cell survival as a percentage of the group treated only in embryo medium. The 5 dpf zebrafish larvae groups were respectively treated with 150 μM Bendavia for 1 hour, 200 μM GT for 1 hour and 150 μM Bendavia for 1 hour followed by 200 μM GT for 1 hour or 2 μM GT for 6 hours (one-way ANOVA, $^{***}P < 0.001$) [Colour figure can be viewed at wileyonlinelibrary.com]

loading of FM1-43FX to 64.9 ± 17.1% of controls ($P < 0.001$; Figure 7), indicating inhibition of MET activity.

4 | DISCUSSION

A lot of evidence suggests that MET channels were the main transporters for the entry of aminoglycosides into inner hair cells (Hailey, Esterberg, Linbo, Rubel, & Raible, 2017). Accordingly, the compounds that block MET activity and aminoglycoside entry exhibit cytoprotective characteristics, such as amiloride, benzamil, raloxifene and ractopamine. In addition, calcium was known to be a MET activity inhibitor (Coffin, Reinhart et al. 2009; Pan, Waguespack, Schnee, LeBlanc, & Ricci, 2012). The low concentration of gentamicin in chronic exposure that induced significant damage to hair cells in the present study was due to the evaluation of toxicity performed under low calcium and magnesium conditions. Bendavia reduced the total amount of gentamicin entering hair cells via inhibition of MET activity, which seems to account for the protective effect against gentamicin in either acute or chronic exposure. However, the results of the present study demonstrated Bendavia reduced gentamicin uptake in lateral line hair cells, which failed to exert its protective effect on lateral line cells against 100 μM gentamicin (as the same level tested in the uptake analysis). It indicated that other mechanisms were included to rescue hair cells against gentamicin.

The underlying mechanism by which gentamicin damages the inner ear has not yet been fully elucidated. Previous studies demonstrated that aminoglycosides are capable of direct interaction with mitochondrial translation machinery, altering the mitochondrial proton gradient followed by MMP collapse (Dehne, Rauen, de Groot, & Lautermann, 2002; Hobbie et al., 2008). It is found that the effect of Bendavia was ascribed to improving mitochondrial capacity and coupling, thereby increasing mitochondrial plasticity in other mitochondrial dysfunction-related disorders (Birk, Chao, Bracken, Warren, & Szeto, 2014; Szeto, Liu, Soong, & Birk, 2015). Similarly, Bendavia provided its protective effect against gentamicin by improving mitochondrial membrane depolarization (Figure 3). Normally, the important mitochondrial functions, such as regulation of the cell redox state, transport of metabolites, lipid and amino acid metabolism and cell death, are highly dependent on MMP (Solaini, Sgarbi, Lenaz, & Baracca, 2007). Accordingly, MMP of hair cells was initially assessed by mitochondrial vital fluorescent dye DASPEI, which was frequently used in evaluating aminoglycoside-induced hair cell damage.

Some reports indicated that gentamicin activates cell death cascades through a diverse signaling pathway with oxidative stress as the early insult. Bendavia is an attractive mitochondria-targeted antioxidant that appears to reduce the production of toxic ROS and stabilize cardiolipin. Cardiolipin is an important component of the inner mitochondrial membrane (IMM), which plays a crucial role in cell death, particularly when it is oxidized. It is particularly sensitive to oxidative damage due to its high content of unsaturated fatty acid and proximity to the site of ROS production (Paradies, Petrosillo, Paradies, &

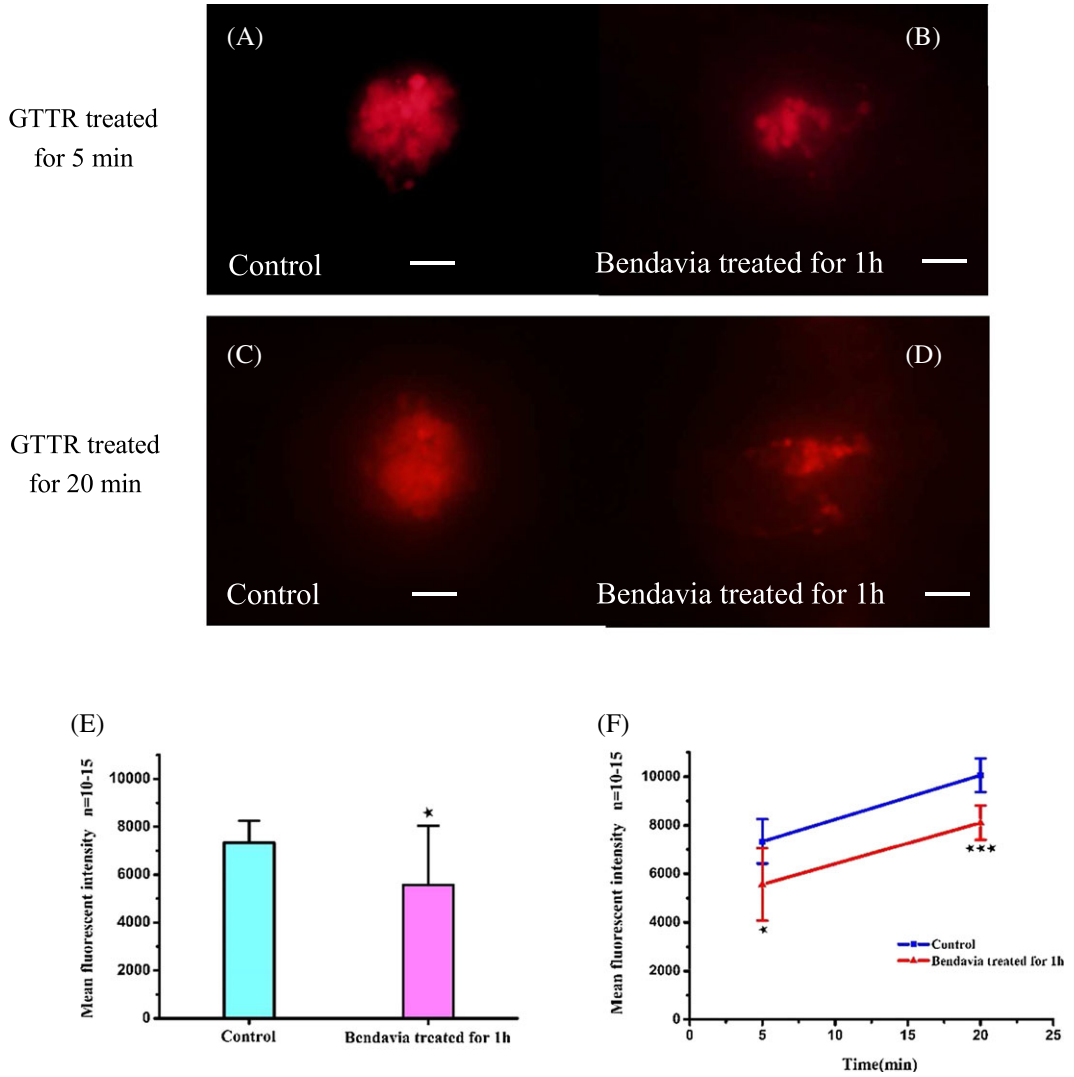

FIGURE 6 In vivo imaging of neuromasts using confocal laser scanning microscopy. Scale bar: 10 μm. Image of neuromasts of 5 dpf larvae, which was immersed in 150 μM Bendavia for 1 hour followed by 100 μM GTTR treated for 5 minutes (B) and 20 minutes (D). Control was treated by 100 μM GTTR only for 5 minutes (A), and 20 minutes (C). Mean fluorescent density of five neuromasts (SO1, SO2, SO3, O1 and O2) was assessed qualitatively per larvae with the form of integral optical density per area. The 5 dpf larvae was treated by 150 μM Bendavia for 1 hour then treated with 100 μM GTTR for 5 minutes (E) and 20 minutes (F) (one-way ANOVA, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). GTTR, gentamicin-conjugated Texas red

Ruggiero, 2011). The oxidative cardiolipin led to cytochrome *c* being detached from the IMM via disruption of supercomplexes and the released cytochrome *c*, in turn, accelerates the oxidation of cardiolipin, resulting in the inhibition of mitochondrial respiration and apoptosis (Szeto, 2014). Bendavia is a new class of molecules that target cardiolipin, which binds to cardiolipin via electrostatic and hydrophobic interactions. Although there is an absence of evidence that less productive ROS exist after pretreatment of Bendavia in the present study, the antioxidant effect of Bendavia should be included when considering its pharmacological outcome.

Antioxidants were recently found to be effective alternative drugs to prevent or cure aminoglycoside-induced hearing loss. For example, SPI-1005 (Ebselen), a synthetic organo-selenium free-radical scavenger, is now being evaluated at the oral dose of 200, 400 and 600 mg twice daily for the prevention and treatment of aminoglycoside-induced ototoxicity in clinical trials (https://clinicaltrials.gov). The drug also showed a positive effect in the treatment of sensorineural hearing loss and Ménière's disease from the Phase II reports. Other antioxidants, including *N*-acetylcysteine, vitamin E, sodium thiosulfate and D-methionine has been evaluated in preclinical or even clinical trials as ROS are supposed to play a critical role in the mediation of ototoxicity (Angeli, Abi-Hachem, Vivero, Telischi, & Machado, 2012; Freyer et al., 2017; Hatano, Uramoto, Okabe, Furukawa, & Ito, 2008; Lindblad, Rosenhall, Olofsson, & Hagerman, 2011; Rewerska, Pawelczyk, Rajkowska, Politanski, & Sliwinska-Kowalska, 2013; Wang et al., 2003). Wu et al. (2015) found *N*-acetylcysteine at a dose of 100 μg ml^{-1} (0.6 mM) significantly prevented the damage of zebrafish lateral hair cells against neomycin and the effect was further improved at a dose of 200 μg ml^{-1} (1.2 mM) dose. It has been proven to be effective against cisplatin-induced ototoxicity in in vivo mammal studies (Saliba, El Fata, Ouelette, & Robitaille, 2010; Thomas Dickey, Muldoon, Kraemer, & Neuwelt, 2004), which promotes further clinical trials (600 mg twice daily systemically) (Riga et al., 2013). Another antioxidant being

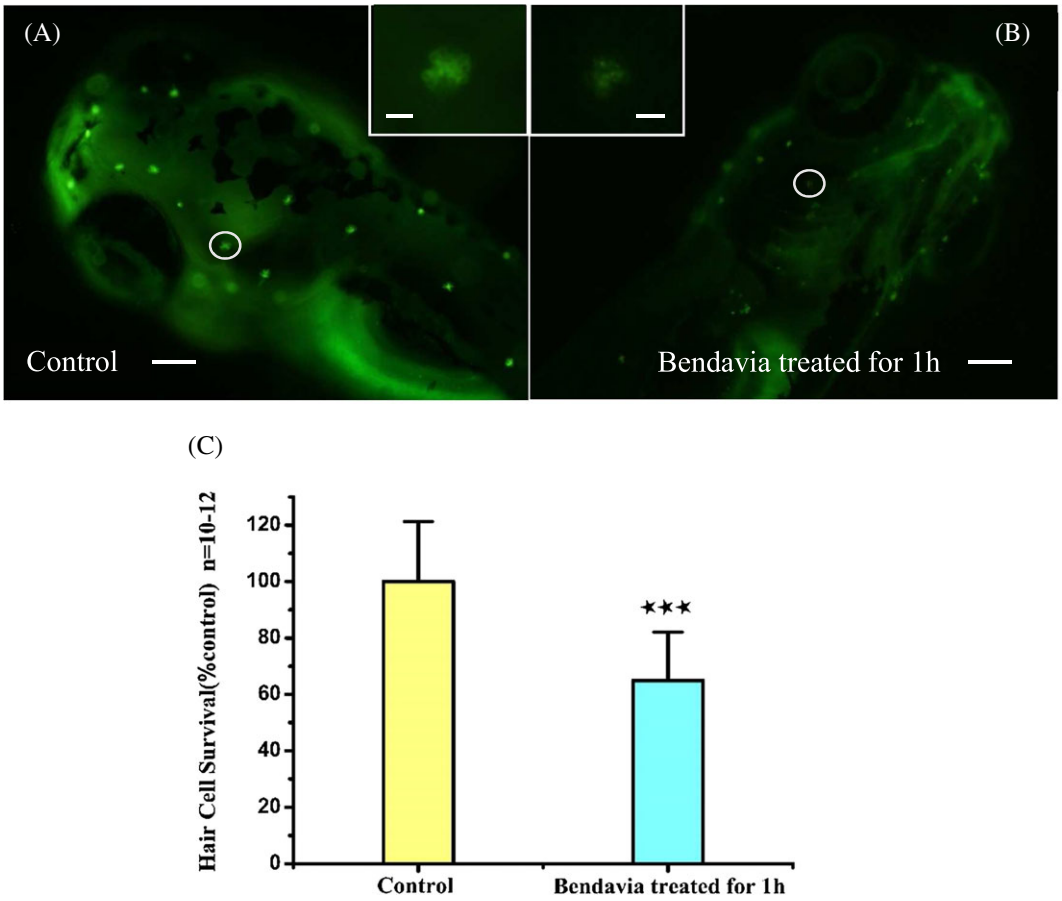

FIGURE 7 Images photographed by fluorescence microscope (scale bar: 200 μm) and the small window next to the zebrafish is an enlarged neuromast O1 stained by FM1-43FX for 45 seconds only (A) or pretreated by Bendavia for 1 hour then FM1-43FX stained (B) in each zebrafish image (Scale bar: 10 μm). Hair cell number was quantified in five neuromasts (SO2, SO3, O1, MI1 and OC1) per larvae, summed to calculate one value per animal and averaged for each group. The 5 dpf zebrafish larvae was treated by 100 μg ml^{-1} Bendavia for 1 hour then FM1-43FX staining for 45 seconds (C). Statistic histogram revealed the conclusion (one-way ANOVA, ***$P < 0.001$)

evaluated in clinical trials against cisplatin-induced ototoxicity is sodium thiosulfate. A Phase III trial reported that intravenous sodium thiosulfate (16 g m^{-2}) provided protection against cisplatin-related hearing loss in children with cancer. However, sodium thiosulfate seems to decrease the survival rate of participants with disseminated disease, indicating the possible tumor-protective effects in addition to being oto-protective (Freyer et al., 2017). In these circumstances, topical administration, such as intratympanic injection, might be an alternative valid approach to delivering therapeutic molecules to the inner ear in the absence of interference with other systemic drugs in vivo (Blakley, 1997). Intratympanic dexamethasone is now being evaluated in Phase IV clinical trial for the treatment of cisplatin-induced ototoxicity (https://clinicaltrials.gov).

Bendavia possesses a highly polar peptide backbone with the presence of a 3+ net charge from two basic amino acids (Lys and Arg) (Zhao, Luo, Zhao, Schiller, & Szeto, 2003). As shown in Figures 6 and 7, it competed with gentamicin uptake into the hair cells through MET channels. MET channels are nonspecific cation channels with a highly electronegative outer surface (Pan et al., 2012), where Bendavia might bind via electrostatic interaction. A detailed mechanism to describe the gating of MET channels needs to be clarified in further research. In addition, no data were to support the interference of Bendavia with aminoglycoside antibiotic efficacy in the present study. Matthew et al. declared that natural bizbenzoquinoline derivatives protected zebrafish lateral line hair cells against aminoglycosides by reducing the uptake of ototoxic drugs with the remaining antibiotic efficacy (Kruger et al., 2016). Owing to the particular MET channels in hair cells, the uptake of aminoglycosides in other cell lines or their antibiotic efficacy in mammal models is required to be determined.

The present investigation demonstrated the feasibility of a novelty antioxidant Bendavia for the protection of hair cells against gentamicin in the zebrafish lateral line system. It was known that hair cells in the zebrafish lateral line exhibit structural and functional similarities to inner ear hair cells of mammals, making it respond to ototoxic agents with a high degree of consistency across the zebrafish model and mammals. Besides, the additional advantages including easy husbandry and high-throughput properties encouraged the following studies to identify potent oto-protectants (Esterberg et al., 2013; MacRae & Peterson, 2015). Nevertheless, the observed protective efficacy in the zebrafish lateral line was unable to guarantee protection in mammalian hair cells due to no separation of inner ear fluid space and stria vascularis in mammals (Uribe, Kawas, Harding, & Coffin, 2015). Therefore, further studies are needed to determine the protection of Bendavia in the mammalian inner ear and to determine appropriateness of concurrent systemic administration with gentamicin.

ACKNOWLEDGEMENTS

This study was financially supported by the National Natural Science Foundation of China (grant no. 81473162) the Program for Liaoning Excellent Talents in University and the Young and middle-aged career development planning of Shenyang Pharmaceutical University.

CONFLICT OF INTEREST

The authors did not report any conflict of interest.

ORCID

Xiao Kuang http://orcid.org/0000-0001-8230-4021

REFERENCES

Angeli, S. I., Abi-Hachem, R. N., Vivero, R. J., Telischi, F. T., & Machado, J. J. (2012). L-N-Acetylcysteine treatment is associated with improved hearing outcome in sudden idiopathic sensorineural hearing loss. *Acta Otolaryngologica*, *132*, 369–376.

Birk, A. V., Chao, W. M., Bracken, C., Warren, J. D., & Szeto, H. H. (2014). Targeting mitochondrial cardiolipin and the cytochrome c/cardiolipin complex to promote electron transport and optimize mitochondrial ATP synthesis. *British Journal of Pharmacology*, *171*, 2017–2028.

Blakley, B. W. (1997). Clinical forum: A review of intratympanic therapy. *American Journal of Otology*, *18*, 520–526. discussion 527–531

Esterberg, R., Coffin, A. B., Ou, H., Simon, J. A., Raible, D. W., & Rubel, E. W. (2013). Fish in a dish: Drug discovery for hearing habilitation. *Drug Discovery Today. Disease Models*, *10*. https://doi.org/10.1016/j.ddmod.2012.02.001

Coffin, A. B., Williamson, K. L., Mamiya, A., Raible, D. W., & Rubel, E. W. (2013). Profiling drug-induced cell death pathways in the zebrafish lateral line. *Apoptosis*, *18*, 393–408.

Coffin, A. B., Reinhart, K. E., Owens, K. N., Raible, D. W., & Rubel, E. W. (2009). Extracellular divalent cations modulate aminoglycoside-induced hair cell death in the zebrafish lateral line. *Hearing Research*, *253*, 42–51.

Courjon, J., Demonchy, E., Degand, N., Risso, K., Ruimy, R., & Roger, P. M. (2017). Patients with community-acquired bacteremia of unknown origin: Clinical characteristics and usefulness of microbiological results for therapeutic issues: A single-center cohort study. *Annals of Clinical Microbiology and Antimicrobials*, *16*, 40.

Daaboul, Y., Korjian, S., Weaver, W. D., Kloner, R. A., Giugliano, R. P., Carr, J., … Gibson, C. M. (2016). Relation of left ventricular mass and infarct size in anterior wall ST-segment elevation acute myocardial infarction (from the EMBRACE STEMI clinical trial). *American Journal of Cardiology*, *118*, 625–631.

Dehne, N., Rauen, U., de Groot, H., & Lautermann, J. (2002). Involvement of the mitochondrial permeability transition in gentamicin ototoxicity. *Hearing Research*, *169*, 47–55.

Freyer, D. R., Chen, L., Krailo, M. D., Knight, K., Villaluna, D., Bliss, B., … Sung, L. (2017). Effects of sodium thiosulfate versus observation on development of cisplatin-induced hearing loss in children with cancer (ACCL0431): A multicentre, randomised, controlled, open-label, phase 3 trial. *Lancet Oncology*, *18*, 63–74.

Hailey, D. W., Esterberg, R., Linbo, T. H., Rubel, E. W., & Raible, D. W. (2017). Fluorescent aminoglycosides reveal intracellular trafficking routes in mechanosensory hair cells. *Journal of Clinical Investigation*, *127*, 472–486.

Harris, J. A., Cheng, A. G., Cunningham, L. L., MacDonald, G., Raible, D. W., & Rubel, E. W. (2003). Neomycin-induced hair cell death and rapid regeneration in the lateral line of zebrafish (Danio Rerio). *Journal of the Association for Research in Otolaryngology*, *4*, 219–234.

Hatano, M., Uramoto, N., Okabe, Y., Furukawa, M., & Ito, M. (2008). Vitamin E and vitamin C in the treatment of idiopathic sudden sensorineural hearing loss. *Acta Otolaryngologica*, *128*, 116–121.

Hobbie, S. N., Akshay, S., Kalapala, S. K., Bruell, C. M., Shcherbakov, D., & Bottger, E. C. (2008). Genetic analysis of interactions with eukaryotic rRNA identify the mitoribosome as target in aminoglycoside ototoxicity. *Proceedings of the National Academy of Sciences U S A*, *105*, 20888–20893.

Hutchin, T., & Cortopassi, G. (1994). Proposed molecular and cellular mechanism for aminoglycoside ototoxicity. *Antimicrobial Agents and Chemotherapy*, *38*, 2517–2520.

Johnson, K., & Messier, S. (2016). Early onset sepsis. *South Dakota Medicine*, *69*, 29–33.

Kezic, A., Spasojevic, I., Lezaic, V., & Bajcetic, M. (2016). Mitochondria-targeted antioxidants: Future perspectives in kidney ischemia reperfusion injury. *Oxidative Medicine and Cellular Longevity*, *2016*. 2950503

Kingwell, K. (2016). First hearing-disorder drugs stumble. *Nature Reviews. Drug Discovery*, *15*, 733–735.

Kruger, M., Boney, R., Ordoobadi, A. J., Sommers, T. F., Trapani, J. G., & Coffin, A. B. (2016). Natural bizbenzoquinoline derivatives protect zebrafish lateral line sensory hair cells from aminoglycoside toxicity. *Frontiers in Cellular Neuroscience*, *10*, 83.

Lanvers-Kaminsky, C., Zehnhoff-Dinnesen, A. A., Parfitt, R., & Ciarimboli, G. (2017). Drug-induced ototoxicity: Mechanisms, Pharmacogenetics, and protective strategies. *Clinical Pharmacology and Therapeutics*, *101*, 491–500.

Lindblad, A. C., Rosenhall, U., Olofsson, A., & Hagerman, B. (2011). The efficacy of N-acetylcysteine to protect the human cochlea from subclinical hearing loss caused by impulse noise: A controlled trial. *Noise and Health*, *13*, 392–401.

Liu, S., Soong, Y., Seshan, S. V., & Szeto, H. H. (2014). Novel cardiolipin therapeutic protects endothelial mitochondria during renal ischemia and mitigates microvascular rarefaction, inflammation, and fibrosis. *American Journal of Physiology: Renal Physiology*, *306*, F970–F980.

Lynch, S. R., & Puglisi, J. D. (2001). Structural origins of aminoglycoside specificity for prokaryotic ribosomes. *Journal of Molecular Biology*, *306*, 1037–1058.

MacRae, C. A., & Peterson, R. T. (2015). Zebrafish as tools for drug discovery. *Nature Reviews Drug Discovery*, *14*, 721–731.

Mishra, A., Mishra, S., Jaganath, G., Mittal, R. K., Gupta, P. K., & Patra, D. P. (1998). Acinetobacter sepsis in newborns. *Indian Pediatrics*, *35*, 27–32.

Ou, H. C., Keating, S., Wu, P., Simon, J. A., Raible, D. W., & Rubel, E. W. (2012). Quinoline ring derivatives protect against aminoglycoside-induced hair cell death in the zebrafish lateral line. *Journal of the Association for Research in Otolaryngology*, *13*, 759–770.

Owens, K. N., Coffin, A. B., Hong, L. S., Bennett, K. O., Rubel, E. W., & Raible, D. W. (2009). Response of mechanosensory hair cells of the zebrafish lateral line to aminoglycosides reveals distinct cell death pathways. *Hearing Research*, *253*, 32–41.

Pan, B., Waguespack, J., Schnee, M. E., LeBlanc, C., & Ricci, A. J. (2012). Permeation properties of the hair cell mechanotransducer channel provide insight into its molecular structure. *Journal of Neurophysiology*, *107*, 2408–2420.

Paradies, G., Petrosillo, G., Paradies, V., & Ruggiero, F. M. (2011). Mitochondrial dysfunction in brain aging: Role of oxidative stress and cardiolipin. *Neurochemistry International*, *58*, 447–457.

Rewerska, A., Pawelczyk, M., Rajkowska, E., Politanski, P., & Sliwinska-Kowalska, M. (2013). Evaluating D-methionine dose to attenuate oxidative stress-mediated hearing loss following overexposure to noise. *European Archives of Otorhinolaryngology*, *270*, 1513–1520.

Riga, M. G., Chelis, L., Kakolyris, S., Papadopoulos, S., Stathakidou, S., Chamalidou, E., … Danielides, V. (2013). Transtympanic injections of N-acetylcysteine for the prevention of cisplatin-induced ototoxicity: A feasible method with promising efficacy. *American Journal of Clinical Oncology*, *36*, 1–6.

Saliba, I., El Fata, F., Ouelette, V., & Robitaille, Y. (2010). Are intratympanic injections of N-acetylcysteine and methylprednisolone protective

against cisplatin-induced ototoxicity? *Journal of Otolaryngology – Head and Neck Surgery*, *39*, 236–243.

Shi, J., Dai, W., Hale, S. L., Brown, D. A., Wang, M., Han, X., & Kloner, R. A. (2015). Bendavia restores mitochondrial energy metabolism gene expression and suppresses cardiac fibrosis in the border zone of the infarcted heart. *Life Sciences*, *141*, 170–178.

Solaini, G., Sgarbi, G., Lenaz, G., & Baracca, A. (2007). Evaluating mitochondrial membrane potential in cells. *Bioscience Reports*, *27*, 11–21.

Szeto, H. H. (2014). First-in-class cardiolipin-protective compound as a therapeutic agent to restore mitochondrial bioenergetics. *British Journal of Pharmacology*, *171*, 2029–2050.

Szeto, H. H., & Birk, A. V. (2014). Serendipity and the discovery of novel compounds that restore mitochondrial plasticity. *Clinical Pharmacology and Therapeutics*, *96*, 672–683.

Szeto, H. H., Liu, S., Soong, Y., & Birk, A. V. (2015). Improving mitochondrial bioenergetics under ischemic conditions increases warm ischemia tolerance in the kidney. *American Journal of Physiology Renal Physiology*, *308*, F11–F21.

Thomas Dickey, D., Muldoon, L. L., Kraemer, D. F., & Neuwelt, E. A. (2004). Protection against cisplatin-induced ototoxicity by N-acetylcysteine in a rat model. *Hearing Research*, *193*, 25–30.

Thomas, A. J., Wu, P., Raible, D. W., Rubel, E. W., Simon, J. A., & Ou, H. C. (2015). Identification of small molecule inhibitors of cisplatin-induced hair cell death: Results of a 10,000 compound screen in the zebrafish lateral line. *Otology & Neurotology*, *36*, 519–525.

Uribe, P. M., Kawas, L. H., Harding, J. W., & Coffin, A. B. (2015). Hepatocyte growth factor mimetic protects lateral line hair cells from aminoglycoside exposure. *Frontiers in Cellular Neuroscience*, *9*, 3.

Wang, J., Lloyd Faulconbridge, R. V., Fetoni, A., Guitton, M. J., Pujol, R., & Puel, J. L. (2003). Local application of sodium thiosulfate prevents cisplatin-induced hearing loss in the guinea pig. *Neuropharmacology*, *45*, 380–393.

Wang, Q., & Steyger, P. S. (2009). Trafficking of systemic fluorescent gentamicin into the cochlea and hair cells. *Journal of the Association for Research in Otolaryngology*, *10*, 205–219.

Wu, C. Y., Lee, H. J., Liu, C. F., Korivi, M., Chen, H. H., & Chan, M. H. (2015). Protective role of L-ascorbic acid, N-acetylcysteine and apocynin on neomycin-induced hair cell loss in zebrafish. *Journal of Applied Toxicology*, *35*, 273–279.

Zhao, K., Luo, G., Giannelli, S., & Szeto, H. H. (2005). Mitochondria-targeted peptide prevents mitochondrial depolarization and apoptosis induced by tert-butyl hydroperoxide in neuronal cell lines. *Biochemical Pharmacology*, *70*, 1796–1806.

Zhao, K., Luo, G., Zhao, G. M., Schiller, P. W., & Szeto, H. H. (2003). Transcellular transport of a highly polar 3+ net charge opioid tetrapeptide. *Journal of Pharmacology and Experimental Therapeutics*, *304*, 425–432.

How to cite this article: Kuang X, Sun Y, Wang Z, Zhou S, Liu H. A mitochondrial targeting tetrapeptide Bendavia protects lateral line hair cells from gentamicin exposure. *J Appl Toxicol*. 2018;38:376-384. https://doi.org/10.1002/jat.3547