Otology & Neurotology
36:519–525 © 2014, Otology & Neurotology, Inc.

Identification of Small Molecule Inhibitors of Cisplatin-Induced Hair Cell Death: Results of a 10,000 Compound Screen in the Zebrafish Lateral Line

*Andrew J. Thomas, *Patricia Wu, *†David W. Raible, *‡Edwin W. Rubel, ||Julian A. Simon, and *‡¶Henry C. Ou

**VM Bloedel Hearing Research Center, †Department of Biological Structure, and ‡Department of Otolaryngology-HNS, University of Washington; ||Fred Hutchinson Cancer Research Center; and ¶Seattle Children's Hospital, Seattle, Washington, U.S.A.*

Hypothesis: The zebrafish lateral line can be used to identify small molecules that protect against cisplatin-induced hair cell death.
Background: Cisplatin is a commonly used chemotherapeutic agent, which causes hearing loss by damaging hair cells of the inner ear. There are currently no FDA-approved pharmacologic strategies for preventing this side effect. The zebrafish lateral line has been used successfully in the past to study hair cell death and protection.
Methods: In this study, we used the zebrafish lateral line to screen a library of 10,000 small molecules for protection against cisplatin-induced hair cell death. Dose-response relationships for identified protectants were determined by quantifying hair cell protection. The effect of each protectant on uptake of a fluorescent cisplatin analog was also quantified.
Results: From this screen, we identified 2 compounds exhibiting dose-dependent protection: cisplatin hair cell protectant 1 and 2 (CHCP1 and 2). CHCP1 reduced the uptake of a fluorescent cisplatin analog, suggesting its protective effects may be due to decreased cisplatin uptake. CHCP2 did not affect uptake, which suggests an intracellular mechanism of action. Evaluation of analogs of CHCP2 revealed 3 additional compounds that significantly reduced cisplatin-induced hair cell death, although none exceed the effectiveness or potency of the parent compound.
Conclusion: The zebrafish lateral line was used to identify 2 small molecules that protected against cisplatin-induced hair cell death. **Key Words:** Cisplatin—Hair cell—Ototoxicity—Protection.

Otol Neurotol **36:**519–525, 2015.

Cisplatin is a commonly used anticancer drug in the treatment of many cancers, including lung cancer, ovarian cancer, and head and neck cancer. It also causes significant nephrotoxicity and ototoxicity. Because cisplatin therapy is administered in planned intervals, it is feasible to administer a protective drug as a cotreatment. Unfortunately, there are currently no FDA-approved drugs that can be used to prevent these organ toxicities. Because of the technical challenges associated with screening mature mammalian hair cells, our group previously developed a method for screening compounds for hair cell toxicity and protection using the lateral line system of free-swimming zebrafish larvae (1). The lateral line contains mechanosensory hair cells that structurally and functionally resemble the hair cells of the inner ear but is easily accessible to drug treatment and imaging as it is located on the exterior of the fish. The lateral line hair cells also exhibit similar susceptibility to known ototoxins including cisplatin and aminoglycosides and have been used to study the hair cell toxicity of these compounds (2–5). We have previously used this method of screening to identify protectants against aminoglycoside hair cell toxicity (1,6). These protectants subsequently demonstrated protection of mammalian hair cells, validating this screening method for discovering potential protectants of mammalian inner ear hair cells (7). In addition, Vlasits et al. (8) used the zebrafish lateral line to screen a library of FDA-approved drugs (Enzo 640) and identified 2 drugs, paroxetine and benzamil, which protected against cisplatin-induced hair cell death.

We screened the ActiProbe 10K (TimTec LLC, Newark, DE, USA) library of 10,000 drug-like small molecules. Small molecules are low–molecular weight organic compounds that have no previously known activity. Small

Address correspondence and reprint requests to Henry C. Ou, M.D., University of Washington, VMBHRC, Box 357923, Bldg CHDD, Rm CD 176, Seattle WA 98195-7923; E-mail: henryou@u.washington.edu
Funding sources: NIH/NIDCD:K08DC009631, P30-DC004661, R01-DC009807, and R01-DC005987-05.

molecules are frequently used in high-throughput screening protocols and can be developed into therapeutic agents but have not yet been developed into drugs. To our knowledge, this is the first small molecule screen for cisplatin protectants described in the literature. From this screen, we identified and characterized 2 compounds with promising activity against cisplatin-induced hair cell death, cisplatin hair cell protectant 1 and 2 (CHCP1 and CHCP2). Although still not at the point of clinical use, these types of protective compounds and the use of high-throughput drug screens to identify them have the potential to lead to clinically useful protective drugs that can one day be used topically or systemically to protect the inner ear.

MATERIALS AND METHODS

Animals

Zebrafish (*Danio rerio*) embryos were produced by paired matings of AB wild-type adult fish maintained at 28.5°C at the University of Washington zebrafish facility (9). Embryos were maintained in fish embryo media (EM; 1 mM $MgSO_4$, 120 μM KH_2PO_4, 74 μM Na_2HPO_4, 1 mM $CaCl_2$, 500 μM KCl, 15 μM NaCl, and 500 μM $NaHCO_3$ in dH_2O) at a density of 50 animals per 100-mm^2 Petri dish and kept in an incubator at 28.5°C. At 4 days postfertilization (dpf), larvae were fed live paramecia. All zebrafish protocols were approved by the University of Washington Institutional Animal Care and Use Committee.

Drug Library

The ActiProbe 10K (TimTec LLC) library of 10,000 drug-like small molecules was screened. The library consisted of 125 96-well plates of 80 compounds per plate. All compounds were provided as 0.05 mg in 50 μl of dimethyl sulfoxide (DMSO). The average molarity of the solutions was 3.0745 mM. A full list of compounds comprising this library is available from TimTec (www.TimTec.com). All compounds in this library are compliant with Lipinski rules (10) to maximize potential for bioavailability.

Initial Screen

Zebrafish larvae were labeled with YO-PRO1 (2 μM × 45 min; Invitrogen, Eugene, OR, USA), a vital fluorescent dye that selectively labels hair cell nuclei. Labeled fish were then transferred in a volume of 148.5 μl EM, one per well, into a Nunc 96-well optical bottom plate (Thermo Fisher Scientific, Waltham, MA, USA); 1.5 μl of compound solution from the library were then added to each corresponding well for a 1:100 dilution (in 1 % DMSO) and average molarity of 6.149 μM (average molecular weight 325.258 g/mol). Fish were incubated with the compounds (or 1% DMSO only controls in EM) for 1 hour. Cisplatin was then added to each well (with the exception of the negative control wells) for a final cisplatin concentration of 50 μM. The fish were incubated for 24 hours and then anesthetized with MS-222 (3-aminobenzoic acid ethyl ester, methanesulfonate salt; Sigma-Aldrich, St. Louis, MO, USA) before imaging. Of the 96 wells, 80 contained cisplatin and one of the test molecules, 8 were used for negative controls (no cisplatin), and 8 were used for positive controls (cisplatin only).

Imaging

For screening, fluorescence microscopy was performed using a Marianas imaging system (Intelligent Imaging Innovations, Denver, CO, USA) incorporating an Axiovert 200M inverted microscope (Zeiss, Thornwood, NY, USA). Bright field visualization of heartbeat and blood flow was used to confirm fish viability. Using a FITC filter, hair cell nuclei labeled with YO-PRO1 dye were observed under a 20X objective for nuclear changes associated with injury. Each fish was assigned a score of 0 to 2, based on an established system (1), with 0 being equivalent to the positive control (complete or nearly complete loss of hair cells, cisplatin 50 μM) and 2 being the negative control (undamaged hair cells, 1% DMSO).

Dose-Response Testing

Wells with a score of 1 or 2 in the initial screen were considered to contain potential protectants and were thus retested. Compounds with a score of 1 or 2 on repeat testing underwent further evaluation with dose-response curves.

Larvae (n = 10 per group) were incubated for 1 hour with each compound at concentrations of 0.5, 1, 5, 10, 20, and 50 μM. Larvae were then treated with cisplatin, 50 μM for 24 hours (protectant present). The larvae were then anesthetized with MS-222 and fixed with 4% paraformaldehyde overnight at 4°C.

After fixation, 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 to 2 hours at room temperature. Larvae were then incubated overnight at 4°C in anti-parvalbumin primary antibody (monoclonal, 1:400 in 1% Triton-X, 1% NGS, in PBS; Millipore, Billerica, MA, USA), then rinsed in 1% Triton-X in PBS (PBS-T) and transferred to Alexa 488 goat antimouse fluorescent secondary antibody solution (1:500, in 1% Triton-X, 1% NGS, in PBS; Invitrogen) for a 2- to 4-hour incubation at room temperature. The larvae were rinsed, then mounted for imaging. A Zeiss Axioplan II microscope with a FITC filter was used to count hair cells from the SO1, SO2, O1, and OC1 neuromasts (11). Approximately 10 fish were counted per group. Results were calculated as the mean hair cell survival as a percentage of the group treated only in EM (negative control).

Rho-Pt Uptake

To determine the effect of each protectant on cisplatin uptake into hair cells, we performed live imaging of uptake of a fluorescent platinum analog (Rho-Pt; Kreatech Diagnostics, Amsterdam, The Netherlands) into lateral line hair cells. We have previously characterized the toxicity and uptake kinetics of Rho-Pt, validating its use as a proxy for cisplatin uptake in the zebrafish lateral line (12). To assess Rho-Pt uptake, 5-dpf zebrafish larvae were pretreated with vehicle only (1.1% DMSO in EM) or 50 μM of the protective compound CHCP1 or CHCP2 (in 1.1% DMSO in EM) for 1 hour. Larvae were then cotreated with 50 μM Rho-Pt for 1 hour and then rinsed in EM. The larvae were then treated with SYTOX Green (5 μM for 1 min; Invitrogen) to label neuromast hair cell nuclei, rinsed in EM and anesthetized before imaging.

Rho-Pt uptake was imaged live in anesthetized larva within a chamber slide containing 2 ml of 0.001% MS-222 in EM. Approximately 5 neuromasts were imaged per larva. Image stacks were obtained using SlideBook 5.0.0.27 x64 software (Intelligent Imaging Innovations) running a Marianas Spinning Disk Confocal system (an Observer.Z1 inverted microscope; Zeiss). A 561-nm laser was used for visualization of Rho-Pt. Optical volumes collected with a Zeiss 63x/1.2W C-Apochromat water-immersion objective were analyzed using Fiji software (13). Rho-Pt uptake in the entire neuromast was measured from a summed intensity projection of the neuromast after correction for specimen background intensities.

Structural Analog Evaluation

Structural analogs of the parent compound CHCP2 were obtained from TimTec by performing a similarity search using the parent compound structure as the query. Three structural analogs were evaluated by analysis of the dose-response relationship against 50 μM cisplatin.

In Vitro Cancer Cell Studies

To evaluate whether the compounds identified as protective against cisplatin toxicity in hair cells altered the chemotherapeutic efficacy of cisplatin, we tested the compounds with cisplatin against human cancer cells in tissue culture. Human lung adenocarcinoma cell lines A549 (ATCC catalog #CCL-185) and NCI-H23 (H23; ATCC catalog #CRL-5800) were used for these experiments. A549 cells were cultured in Dulbecco's modified Eagle medium high glucose (DMEM; catalog SH30022.01; Thermo Scientific, Waltham, MA, USA) with 10% fetal bovine serum (FBS; Mediatech, Inc., Manassas, VA, USA), and 1% L-glutamine (Invitrogen, Grand Island, NY, USA). NCI-H23 cells were cultured in RPMI-1640 medium (containing 10 mM HEPES, 1% L-glutamine, and sodium bicarbonate; Thermo Scientific) with 10% FBS and 1% sodium pyruvate (Thermo Scientific). Cells were diluted to a concentration of 5,000 per ml and transferred to Costar 3917 assay plates (Corning, Inc., Corning, NY, USA) in 100 μL of media for 500 cells per well. Incubations were performed at 37° C in a humidified, 5% CO_2 incubator. The duration of incubations differed between the cell lines because of difference in doubling times; A549 cells were incubated for 6 hours, and H23 cells for 24 hours, to allow them to adhere to the culture plate. A matrix of combinations of cisplatin at 0, 5, 10, and 20 μM, with CHCP1 at 0, 5, 10, and 20 μM or CHCP2 at 0, 5, 10, and 20 μM, was used with 4 wells for each combination. After addition of protective compounds and cisplatin, the plates were incubated for 48 hours. The treatment-containing medium was then removed from each well and replaced with 100 μl of the appropriate growth medium, and the cells were incubated (A549 cells for 16 hours, H23 cells for 24 hours) to allow them to recover. Medium was then removed from each well and replaced with 50 μl of the appropriate medium with a reduced FBS concentration of 2%. Cell viability was measured using the CellTiter-Glo luminescent cell viability assay (Promega, Madison, WI, USA). Cell luminescence was assessed with a TopCount NXT microplate luminescence counter (Packard Instrument Company, Meriden, CT, USA).

Statistical Analysis

Comparison of mean hair cell survival between multiple concentrations of each protectant compound was accomplished using 1-way analysis of variance (ANOVA) with Tukey's post hoc test for individual comparisons. For experiments with multiple groups (tumor cell culture), a 2-way ANOVA with a Bonferroni posttest was used to compare means. A threshold of $p < 0.05$ was used for statistical significance. Statistical tests were performed using GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego, CA, USA).

RESULTS

Small Molecules That Protect Against Cisplatin-Induced Hair Cell Death

In our screen of the ActiProbe 10K library, we identified 2 compounds that reliably protected against cisplatin-induced hair cell death. These 2 compounds are referred to here as cisplatin hair cell protectant 1 (CHCP1), ethyl 4-{[(2,4-dinitrophenyl)amino]azamethylene}-2,5-dimethylpiperidinecarboxylate, and cisplatin hair cell protectant 2 (CHCP2), 6-methyl-3-(3,3,7,8-tetramethyl-1-oxo(2,3,4-trihydro-5H,10H,11H-benzo{b}benzo[2,1-f]1,4-diazepin-11-yl))chromen-4-one (Fig. 1A).

Dose-response titrations were performed to further evaluate the protective effect of these compounds. Pretreatment for 1 hour with 25 μM CHCP1, followed by 24 hours of cotreatment with 50 μM cisplatin significantly increased

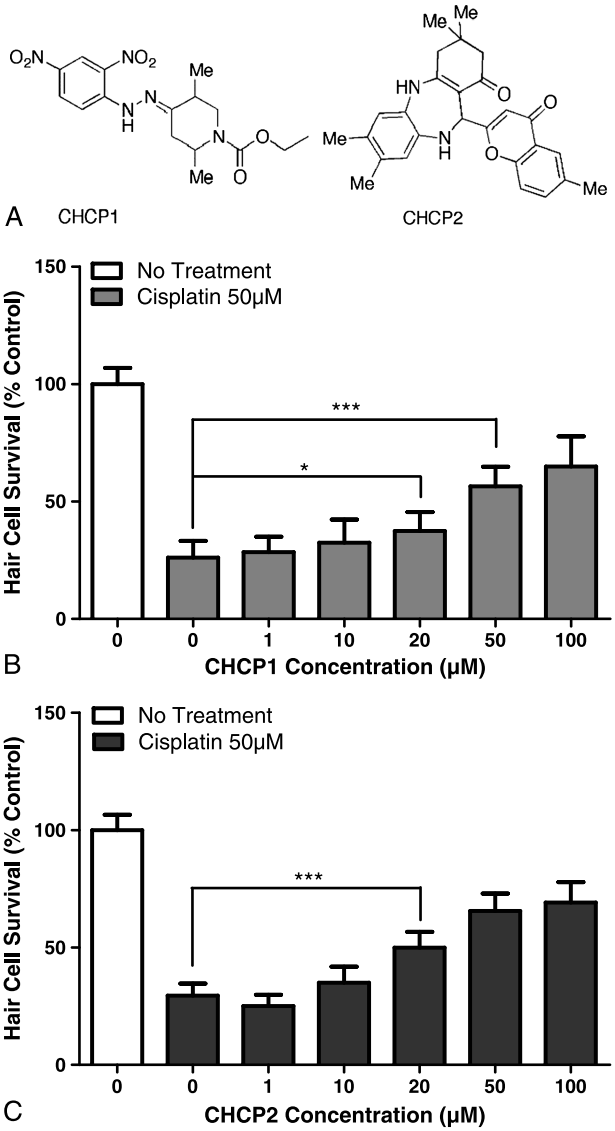

FIG. 1. CHCP 1 and 2 cause concentration-dependent reduction in cisplatin-induced hair cell death. *A*, Chemical structures of CHCP1 and CHCP2. Dose-response functions for cotreatment with 50 μM cisplatin and either (*B*) CHCP1 or (*C*) CHCP2 for 24 h, demonstrating an increase in hair cell survival, which reached significance at a concentration of 20 μM of the protectant ($p < 0.05$ for CHCP1 and $p < 0.001$ for CHCP2, Tukey-Kramer posttest). For all treatment groups, n = 7 to 13 fish. Error bars = ±1 SD; ***$p < 0.001$, *$p < 0.05$, $^{ns}p > 0.05$ by 1-way ANOVA and Tukey-Kramer posttest.

hair cell survival from 37.11 ± 3.99% (cisplatin-only controls) to 61.86 ± 9.69% (Fig. 1B; $p < 0.001$, 1-way ANOVA with Tukey-Kramer posttest). No significant increase in protection was observed after surpassing a concentration of 50 μM, and systemic toxicity was observed at higher concentrations (data not shown).

Pretreatment for 1 hour with 100 μM CHCP2, followed by 24 hours of cotreatment with 50 μM cisplatin significantly increased hair cell survival from 29.74 ± 5.26% (cisplatin-only controls) to 70.00 ± 10.24% (Fig. 1C; $p < 0.001$, 1-way ANOVA with Tukey-Kramer posttest). Although 100 μM of CHCP2 represented the maximum protection observed, the increase in hair cell survival between doses of 50 μM (65.19 ± 7.84%) and 100 μM was not significant. No signs of toxicity were observed at 150 μM, the highest concentration of CHCP2 evaluated.

We then examined whether the protection by a fixed dose of CHCP1 or CHCP2 would be maintained at higher doses of cisplatin. We found that 50 μM CHCP1 resulted in significant protection against 25 and 50 μM cisplatin ($p < 0.05$, 2-way ANOVA and Tukey-Kramer posttest) but not against higher doses of cisplatin (Fig. 2A). In contrast, 50 μM CHCP2 maintained protection against 75 and 100 μM cisplatin ($p < 0.01$, 2-way ANOVA, Tukey-Kramer posttest; Fig. 2B).

CHCP1 Reduced Uptake of Fluorescent Platinum Analog Rho-Pt

We used rhodamine-conjugated cisplatin (Rho-Pt) to evaluate whether CHCP1 or CHCP2 might protect hair cells by affecting cisplatin uptake. CHCP1 treatment resulted in significant reduction of Rho-Pt fluorescence to 58.95 ± 33.83% of controls ($p < 0.05$, 1-way ANOVA with Tukey-Kramer posttest). CHCP2 treatment resulted in Rho-Pt fluorescence of 121.87 ± 40.86%, which was not significantly different from controls (Fig. 3).

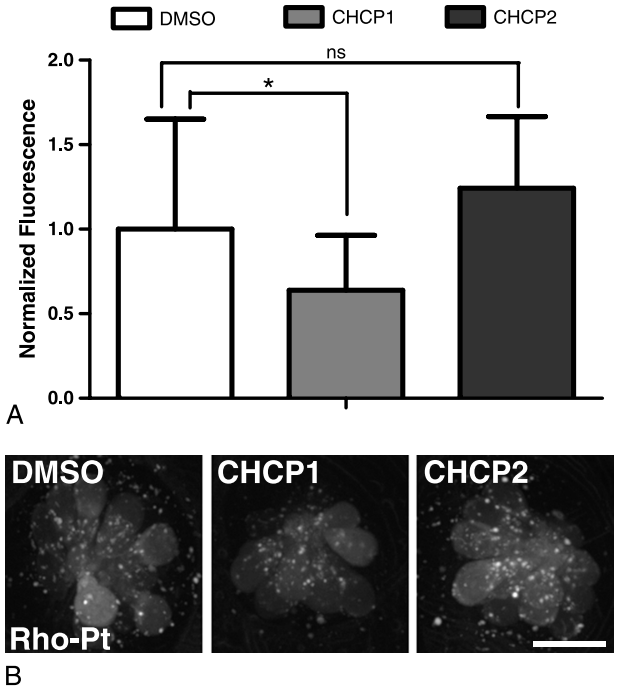

FIG. 3. Treatment with CHCP1, but not CHCP2, reduces hair cell uptake of Rho-Pt. *A*, Quantified Rho-Pt fluorescence after 1 hour of treatment with the indicated protective compound normalized to untreated controls. Rho-Pt uptake is significantly reduced with CHCP1 treatment but unchanged with CHCP2. *B*, Representative neuromasts, which received pretreatment and cotreatment with a protective compound or DMSO (control) as indicated. Rho-Pt uptake labeling of hair cells is reduced with CHCP1 compared with DMSO control and CHCP2. Scale bar for all figures = 10 μm. Error bars = ±1 SD; n = 22–35 neuromasts per treatment group; $^{ns}p > 0.05$, $^{*}p < 0.05$, by 1-way ANOVA and Tukey-Kramer posttest.

Both CHCP1 and CHCP2 Have Small but Significant Effects on the Tumoricidal Efficacy of Cisplatin

For a systemically administered protectant against cisplatin ototoxicity to be therapeutically useful, it should not prevent the chemotherapeutic action of cisplatin. To begin evaluating this issue, we cultured A549 and NCI-H23 human lung adenocarcinoma cells and assessed the efficacy of cisplatin in combination with different concentrations of protectant compound (Fig. 4). Cisplatin

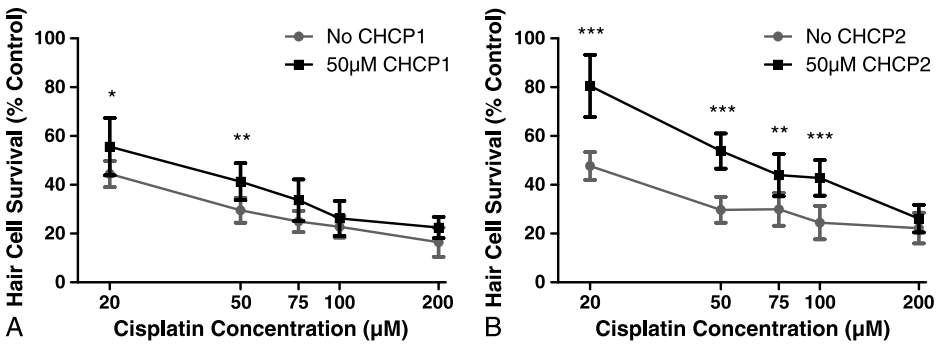

FIG. 2. The protective effects of CHCP 1 and 2 are only partially maintained with increased cisplatin concentration. *A*, Cotreatment with 50 μM CHCP1 results in a significant increase in hair cell survival at cisplatin concentrations up to 50 μM ($p < 0.01$, Tukey-Kramer posttest). *B*, Cotreatment with 50 μM CHCP2 results in a significant increase in hair survival at cisplatin concentrations up to 100 μM ($p < 0.001$, Tukey-Kramer posttest). For all treatment groups, n = 9–12 fish. Error bars = ±1 SD; $^{***}p < 0.001$, $^{**}p < 0.01$, and $^{*}p < 0.05$, by 1-way ANOVA and Tukey-Kramer posttest.

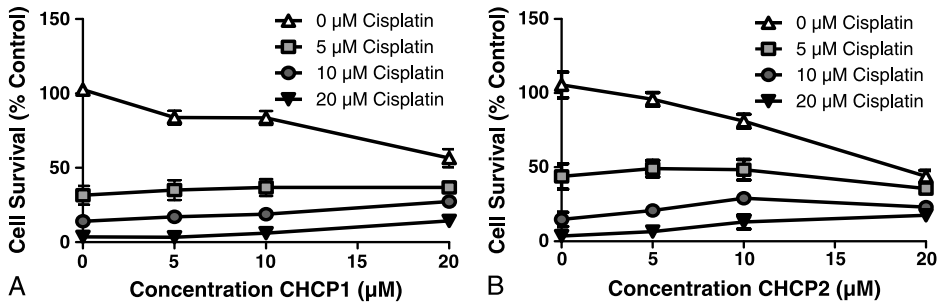

FIG. 4. Effect of CHCP1 and CHCP2 on cytotoxic activity of cisplatin. Cytotoxic activity of cisplatin is maintained in the presence of protective compounds at some doses of cisplatin but attenuated at others. *A*, CHCP1 demonstrated a small but significant inhibition of cisplatin toxicity in A549 cancer cells at 10 and 20 μM cisplatin ($p < 0.001$, 1-way ANOVA). At 5 μM cisplatin, there was no significant inhibition of tumor cell kill. CHCP1 alone (0 μM cisplatin) independently had significant toxicity to tumor cells. *B*, CHCP2 also demonstrated a small but significant inhibition of cisplatin toxicity in A549 cancer cells at 10 and 20 μM cisplatin ($p < 0.001$, 1-way ANOVA). At 5 μM cisplatin, there was no significant inhibition of tumor cell kill. CHCP2 alone (0 μM cisplatin) independently had significant toxicity to tumor cells. Similar results were obtained when these experiments were repeated with NCI-H23 cells (data not shown). Each condition was performed in quadruplicate. Data points are the mean A549 cancer cell survival as determined by ATP cell luminescence assay. Error bars = ±1 SD.

concentrations of 0, 5, 10, and 20 μM were used, resulting in tumor cell survival of roughly 100%, 35%, 10%, and 2% of untreated controls, respectively. For 5 μM cisplatin, dose-dependent cisplatin-induced kill of tumor cells was maintained across the concentrations of CHCP1 and 2 evaluated. For the 10- and 20-μM cisplatin doses, there was a small but significant reduction in tumor kill with increasing doses of CHCP1 and CHCP2 ($p < 0.001$, 1-way ANOVA). In addition, both CHCP1 alone and CHCP2 alone caused dose-dependent tumor cell death ($p < 0.05$, 1-way ANOVA).

Structural Analogs of CHCP2 Protect Against Cisplatin-Induced Hair Cell Death

Three structural analogs of CHCP2 (CHCP 2.1, 2.2, and 2.3; Fig. 5A) were evaluated, and all demonstrated significant protection against cisplatin-induced hair cell death (Fig. 5B; $p < 0.001$, 1-way ANOVA and Tukey-Kramer posttest). However, none of the 3 structural analogs demonstrated significantly improved efficacy or potency from CHCP2. Additionally, CHCP2.1 began to show toxicity at 20 μM, and thus, higher concentrations could not be assessed. At 20 μM, there was no significant difference between protection from CHCP2 and CHCP2.3. CHCP2.1 and CHCP2.2 demonstrated significantly less protection than CHCP2 ($p < 0.05$ and $p < 0.001$, respectively, 1-way ANOVA and Tukey-Kramer posttest).

DISCUSSION

Identification of Small Molecule Inhibitors of Cisplatin-Induced Hair Cell Death

To our knowledge, this is the first screen of a small molecule library for inhibitors of cisplatin-induced hair cell death. Although these small molecule protectants are promising, they must undergo ''hit expansion'' (testing of structural variants) and lead optimization (optimizing specificity and potency) en route to the development of a possible drug for human use. Nevertheless, this study demonstrates the feasibility of using a phenotypic screen to identify small molecule protectants against cisplatin-induced hair cell death. This screening method has the potential to provide a pipeline of candidate protectants that can protect hair cells against damage and could someday reach clinical use. The ability to rapidly identify a large number of protective drugs is particularly important in light of findings that inhibition of one cell death pathway can lead to upregulation of others. This makes it less likely that a single drug will be able to provide complete protection of the inner ear and makes the use of protective ''cocktails'' involving multiple protectants potentially more valuable.

Comparison of CHCP1 to CHCP2

Of the 2 candidate protectants, CHCP2 is more promising for a number of reasons. Functionally, in our testing, CHCP2 had a lower ED50 for hair cell protection and a better range for hair cell survival than CHCP1. In addition, CHCP2 did not have any obvious systemic toxicity, whereas doses of CHCP1 above 100 μM began to be lethal to the zebrafish larvae. CHCP2 was also able to maintain protection over a wider range of cisplatin doses, with protection maintained against 100 μM cisplatin for 24 hours.

Structurally, CHCP2 has multiple favorable characteristics compared with CHCP1. CHCP1 contains 2 nitro ($-NO_2$) groups that are prone to undergo hepatic metabolism generating reactive intermediates (14) and can lead to toxicity. In addition, the central hydrazone linkage (C=N–N) is susceptible to acid-catalyzed hydrolysis, making CHCP1 potentially less stable. Hydrolysis of the hydrazone linkage also generates hydrazines (N_2H_3R) that can have significant toxicity.

In contrast, CHCP2 fits well with the ''Lipinski rules'' that predict how ''drug-like'' a compound is (15). It has no reactive sites that might lead to instability or toxicity and would be predicted to have good bioavailability. The structure of CHCP2 uses a benzodiazepine scaffold,

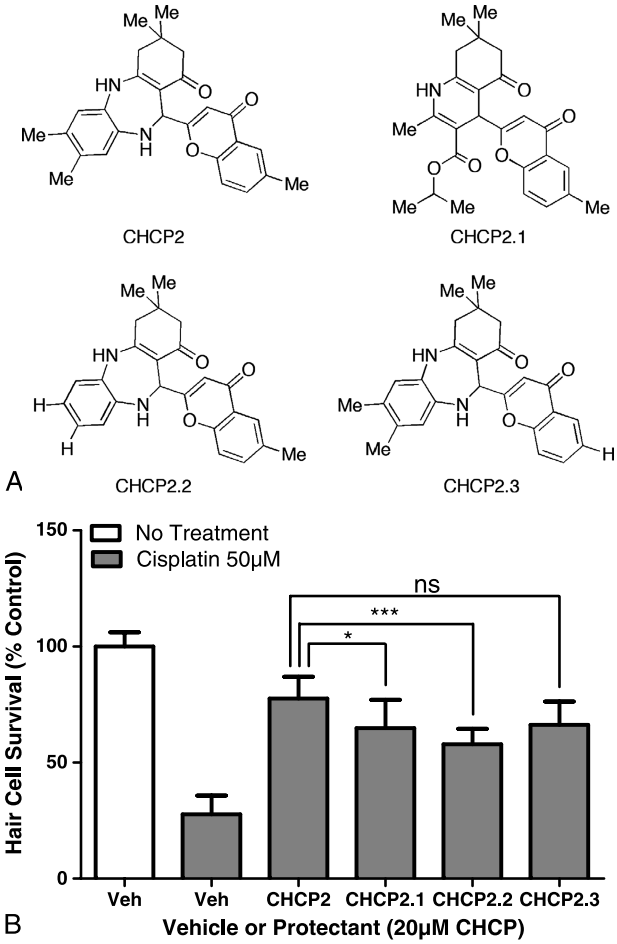

FIG. 5. Analogs of CHCP2 protect against cisplatin-induced hair cell death. *A*, Structures of CHCP2 and CHCP2 analogs. *B*, At a concentration of 20 μM, all 3 CHCP analogs demonstrate significant protection against cisplatin-induced hair cell death compared with DMSO-treated controls. CHCP2.1 and 2.2 exhibit significantly less protection than CHCP2, whereas 2.3 achieves similar protection as CHCP2. For all treatment groups, n = 8 to 11 fish. Error bars = ±1 SD; $^{***}p < 0.001$, $^{*}p < 0.05$, $^{ns}p > 0.05$ by 1-way ANOVA and Tukey-Kramer posttest.

considered one of the ''privileged scaffolds'' for biologically active compounds (16).

Mechanisms of Protection—Uptake Inhibition Versus Intracellular Mechanisms

We recently characterized Rho-Pt as a fluorescent analog of cisplatin that can be used to examine cisplatin uptake (12). We found that like aminoglycoside uptake, uptake of Rho-Pt into hair cells required functional mechanotransduction. Here, we used Rho-Pt to examine whether the protective compounds might act by blocking uptake of cisplatin into the hair cells. We found that CHCP1 but not CHCP2 significantly reduced cisplatin uptake relative to controls, suggesting that CHCP1 might interfere with mechanotransduction. On the other hand, we did not find that it protected against aminoglycoside-induced hair cell death (data not shown). CHCP1 had significant systemic toxicity to the zebrafish at higher concentrations, and although there was no hair cell death, it is possible that reduced hair cell health could actually be contributing to the reduction in Rho-Pt uptake.

In contrast, the lack of significant reduction in Rho-Pt uptake in the presence of CHCP2 suggests that this compound is exerting its protective effects by acting on intracellular targets and not on uptake mechanisms. Intracellular mechanisms involved in cisplatin-induced hair cell toxicity include production of ROS (17,18), damage to mitochondria, STAT-1 activation (19), and activation of caspases (20,21). In addition, it has been demonstrated that inhibition of one cell-death pathway can lead to activation of others (22–25). This is likely the reason several compounds that target intracellular mechanisms of ototoxicity have achieved partial protection (21,26,27).

Effect of Protectants on Cisplatin Chemotherapeutic Efficacy

At several cisplatin doses, both CHCP1 and CHCP2 demonstrated small but significant inhibition of cisplatin-induced tumor cell kill in A549 and NCI-H23 cell lines. In both cases, at other doses of cisplatin, there was no inhibition of tumor cell kill. Interestingly, when tested alone without cisplatin, each compound had some cytotoxic effects. However, this effect was not sustained when used in combination with cisplatin and could be interpreted as the protectant interfering with the cytotoxic effects of cisplatin, or vice versa. It should be noted that Vlasits et al. (8) did not find any significant interference with the cisplatin activity in their screen for protective drugs. It remains to be seen whether these findings will be confirmed in vivo.

Ideally, a drug could be given systemically and protect the inner ear while having no impact on (or potentially facilitating) cisplatin-induced tumor cell kill. The challenge of achieving complete protection against hair cell death while preserving tumor cell death is a significant one because there may be overlapping pathways. However, because cisplatin primarily targets dividing cells, and hair cells lack the capacity to proliferate, there likely are significant differences in their death pathways. Alternatively, local application of protectants would achieve high concentration of the drug in the inner ear with little systemic absorption and low drug concentration at the site of tumor. Current work on inner ear drug delivery systems is making this approach increasingly feasible (28–30). In addition, our previous finding that cisplatin uptake may be dependent on functional mechanotransduction (12) may suggest a hair cell–specific mechanism of protection that presumably would not impact cancer cells.

Identifying Analogs With Improved Characteristics

We evaluated structural analogs of CHCP2 in hopes of identifying similar compounds with greater efficacy and potency of protection against cisplatin-induced hair cell death. We also hoped to gain information about the structure-activity relationship of the compound. All 3

analogs demonstrated protection against cisplatin-induced hair cell death, confirming the protective capacity of the underlying structural chemical structure. However, none of the analogs that we evaluated exhibited significantly improved efficacy or potency from the parent compound. Analogs were selected for modifications to particular locations on the parent compound in hopes of gaining information about the functional importance of that region of the molecule. Evaluation of a greater number of structural analogs through systematic chemical modification is needed.

Limitations

Although the zebrafish is an efficient model system for studying hair cell protection, there are limitations. Unlike the mammalian inner ear, there is no division of fluid spaces in the lateral line, with hair cells extending their stereocilia into the surrounding water. There is also no distinction between inner versus outer hair cells. Those limitations aside, the zebrafish allows us to examine hair cell death and protection in an in vivo system, which affords some advantages over more traditional in vitro hair cell systems, such as hair cell lines and whole organ cultures.

CONCLUSION

This study further demonstrates the feasibility of phenotypic screening for hair cell protectants using the zebrafish lateral line system. To our knowledge, this represents the first successful discovery of protectants against cisplatin-induced hair cell death in a small molecule library. Follow-up studies are needed to determine whether similar protection is seen in the mammalian inner ear and to determine appropriateness of concurrent systemic administration with cisplatin. In addition, it is hoped that mammalian studies will also examine protection against other cisplatin side effects including neurotoxicity and nephrotoxicity.

Acknowledgment: The authors thank Eli Ocheltree for the technical expertise and assistance with cell culture.

REFERENCES

1. Owens KN, Santos F, Roberts B, et al. Identification of genetic and chemical modulators of zebrafish mechanosensory hair cell death. *PLoS Genet* 2008;4:e1000020.
2. Williams JA, Holder N. Cell turnover in neuromasts of zebrafish larvae. *Hear Res* 2000;143:171–81.
3. Harris JA, Cheng AG, Cunningham LL, MacDonald G, Raible DW, Rubel EW. Neomycin-induced hair cell death and rapid regeneration in the lateral line of zebrafish (Danio rerio). *J Assoc Res Otolaryngol* 2003;4:219–34.
4. Ton C, Parng C. The use of zebrafish for assessing ototoxic and otoprotective agents. *Hear Res* 2005;208:79–88.
5. Ou HC, Raible DW, Rubel EW. Cisplatin-induced hair cell loss in zebrafish (Danio rerio) lateral line. *Hear Res* 2007;233:46–53.
6. Ou HC, Cunningham LL, Francis SP, et al. Identification of FDA-approved drugs and bioactives that protect hair cells in the zebrafish (Danio rerio) lateral line and mouse (Mus musculus) utricle. *J Assoc Res Otolaryngol* 2009;10:191–203.
7. Coffin AB, Ou H, Owens KN, et al. Chemical screening for hair cell loss and protection in the zebrafish lateral line. *Zebrafish* 2010;7:3–11.
8. Vlasits AL, Simon JA, Raible DW, Rubel EW, Owens KN. Screen of FDA-approved drug library reveals compounds that protect hair cells from aminoglycosides and cisplatin. *Hear Res* 2012;294:153–65.
9. Westerfield M. *The Zebrafish Book: A Guide for the Laboratory Use of Zebrafish (Danio rerio)*. Eugene, OR: University of Oregon Press; 2000.
10. Lipinski CA, Lombardo F, Dominy BW, Feeney PJ. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Adv Drug Deliv Rev* 2001;46:3–26.
11. Raible DW, Kruse GJ. Organization of the lateral line system in embryonic zebrafish. *J Comp Neurol* 2000;421:189–98.
12. Thomas AJ, Hailey DW, Stawicki TM, et al. Functional mechanotransduction is required for cisplatin-induced hair cell death in the zebrafish lateral line. *J Neurosci* 2013;33:4405–14.
13. Schindelin J, Arganda-Carreras I, Frise E, et al. Fiji: an open-source platform for biological-image analysis. *Nat Methods* 2012;9:676–82.
14. Westerfeld WW, Richert DA, Higgins ES. The metabolic reduction of organic nitro groups. *J Biol Chem* 1957;227:379–91.
15. Lipinski CA. Lead- and drug-like compounds: the rule-of-five revolution. *Drug Discov Today: Technol* 2004;1:337–41.
16. Hata M, Marshall GR. Do benzodiazepines mimic reverse-turn structures? *J Comput Aided Mol Des* 2006;20:321–31.
17. Kim HJ, Lee JH, Kim SJ, et al. Roles of NADPH oxidases in cisplatin-induced reactive oxygen species generation and ototoxicity. *J Neurosci* 2010;30:3933–46.
18. Rybak LP, Whitworth CA, Mukherjea D, Ramkumar V. Mechanisms of cisplatin-induced ototoxicity and prevention. *Hear Res* 2007;226:157–67.
19. Schmitt NC, Rubel EW, Nathanson NM. Cisplatin-induced hair cell death requires STAT1 and is attenuated by epigallocatechin gallate. *J Neurosci* 2009;29:3843–51.
20. Liu W, Staecker H, Stupak H, Malgrange B, Lefebvre P, Van De Water TR. Caspase inhibitors prevent cisplatin-induced apoptosis of auditory sensory cells. *Neuroreport* 1998;9:2609–14.
21. Wang J, Ladrech S, Pujol R, Brabet P, Van De Water TR, Puel JL. Caspase inhibitors, but not c-Jun NH2-terminal kinase inhibitor treatment, prevent cisplatin-induced hearing loss. *Cancer Res* 2004;64:9217–24.
22. Boya P, Gonzalez-Polo RA, Casares N, et al. Inhibition of macroautophagy triggers apoptosis. *Mol Cell Biol* 2005;25:1025–40.
23. Vandenabeele P, Vanden Berghe T, Festjens N. Caspase inhibitors promote alternative cell death pathways. *Sci STKE* 2006;2006:pe44.
24. Zhang DW, Shao J, Lin J, et al. RIP3, an energy metabolism regulator that switches TNF-induced cell death from apoptosis to necrosis. *Science* 2009;325:332–6.
25. Zhivotovsky B, Orrenius S. Cell cycle and cell death in disease: past, present and future. *J Intern Med* 2010;268:395–409.
26. Shin YS, Song SJ, Kang S, Hwang HS, Jung YS, Kim CH. Novel synthetic protective compound, KR-22335, against cisplatin-induced auditory cell death. *J Appl Toxicol* 2014;34:191–204.
27. Choi SJ, Kim SW, Lee JB, et al. Gingko biloba extracts protect auditory hair cells from cisplatin-induced ototoxicity by inhibiting perturbation of gap junctional intercellular communication. *Neuroscience* 2013;244:49–61.
28. Salt AN, Plontke SK. Local inner-ear drug delivery and pharmacokinetics. *Drug Discov Today* 2005;10:1299–1306.
29. Swan EE, Mescher MJ, Sewell WF, Tao SL, Borenstein JT. Inner ear drug delivery for auditory applications. *Adv Drug Deliv Rev* 2008;60:1583–99.
30. McCall AA, Swan EE, Borenstein JT, Sewell WF, Kujawa SG, McKenna MJ. Drug delivery for treatment of inner ear disease: current state of knowledge. *Ear Hear* 2010;31:156–65.