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Using the Zebrafish Lateral Line to Screen for Ototoxicity

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ABSTRACT

The zebrafish is a valuable model for studying hair cell development, structure, genetics, and behavior. Zebrafish and other aquatic vertebrates have hair cells on their body surface organized into a sensory system called the lateral line. These hair cells are highly accessible and easily visualized using fluorescent dyes. Morphological and functional similarities to mammalian hair cells of the inner ear make the zebrafish a powerful preparation for studying hair cell toxicity. The ototoxic potential of drugs has historically been uncovered by anecdotal reports that have led to more formal investigation. Currently, no standard screen for ototoxicity exists in drug development. Thus, for the vast majority of Food and Drug Association (FDA)-approved drugs, the ototoxic potential remains unknown. In this study, we used 5-day-old zebrafish larvae to screen a library of 1,040 FDA-approved drugs and bioactives (NINDS Custom Collection II) for ototoxic effects in hair cells of the lateral line. Hair cell nuclei were selectively labeled using a fluorescent vital dye. For the initial screen, fish were exposed to drugs from the library at a 100-μM concentration for 1 h in 96-well tissue culture plates. Hair cell viability was assessed in vivo using fluorescence microscopy. One thousand forty drugs were rapidly screened for ototoxic effects. Seven known ototoxic drugs included in the library, including neomycin and cisplatin, were positively identified using these methods, as proof of concept. Fourteen compounds without previously known ototoxicity were discovered to be selectively toxic to hair cells. Dose–response curves for all 21 ototoxic compounds were determined by quantifying hair cell survival as a function of drug concentration. Dose–response relationships in the mammalian inner ear for two of the compounds without known ototoxicity, pentamidine isethionate and propantheline bromide, were then examined using in vitro preparations of the adult mouse utricle. Significant dose-dependent hair cell loss in the mouse utricle was demonstrated for both compounds. This study represents an important step in validating the use of the zebrafish lateral line as a screening tool for the identification of potentially ototoxic drugs.

Keywords: hair cell, zebrafish, lateral line, drug screen, ototoxicity

INTRODUCTION

Hearing loss affects more than 28 million Americans, including approximately 50% of people more than the age of 75 years and 2% of children (National Institute on Deafness and Other Communication Disorders, NIDCD 2007). Known etiologies of hearing loss include genetic factors, acoustic injury, mechanical trauma, medications, infection, and age-related changes. Most cases of peripheral sensorineural auditory pathology involve some form of cochlear abnormality that disrupts sensory transduction at the level of the auditory nerve, stria vascularis, or hair cell. Hair cells within the organ of Corti in the inner ear are

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highly metabolic and are particularly vulnerable to noxious insults like noise or chemicals. Thus, one of the most common histopathological findings in hearing loss is hair cell loss (Schuknecht 1993).

One common form of ototoxicity is the tendency of drugs and environmental toxins to cause damage to the receptor epithelia of hearing and/or balance. Seligmann et al. (1996) listed more than 130 drugs and chemicals reported to be potentially ototoxic, with the major classes of known ototoxic compounds being the aminoglycosides and other antimicrobials, anti-inflammatory agents, diuretics, antimalarial drugs, antineoplastic agents, and some topically administered agents. Most of these drugs were initially recognized as being ototoxic after anecdotal reports of hearing loss, tinnitus, or balance impairment. These reports subsequently prompted controlled studies in humans or laboratory animals on the ototoxicity of individual drugs. Without the initial anecdotal reports, further research into ototoxicity would not have been conducted, since screening for ototoxicity is not generally included in drug development protocols. At present, there is no standardized screening process for ototoxicity in drug development, and the ototoxic potential of the vast majority of Food and Drug Association (FDA)-approved drugs remains unknown.

Despite a large research effort into the mechanisms and etiologies underlying hearing loss, it is likely that some idiopathic sensorineural hearing loss is secondary to exposure to drugs or chemicals that are not known to be ototoxic. According to the NIDCD, less than 15% of patients with sudden deafness know the causes of their hearing loss (NIDCD 2007). The etiology of sensorineural hearing loss in children is unknown 37.7% of the time (Morzaria et al. 2004) and due to unknown environmental or nongenetic causes 22% of the time (Gurtler and Lalwani 2002). It is reasonable to presume that hearing loss from ototoxic medications is underestimated, especially in pediatric patients, in whom hearing loss is more difficult to detect. Children often may be unable to recognize or communicate the presence of newly acquired hearing loss. Furthermore, ototoxic effects are likely to be misattributed to presbycusis in older patients, who are more likely to be receiving multiple medications. Another contributing factor to the underestimation of sensorineural hearing loss due to ototoxicity is the phenomenon of hair cell loss with an absence of detectable hearing loss on conventional audiometry. For example, conventional pure-tone audiometry can fail to detect hearing loss in children being treated with chemotherapeutic cisplatin derivatives (Stavroulaki et al. 2001; Dhooge et al. 2006; Knight et al. 2007). In summary, it is probable that there is a subset of drugs currently used in practice that have occult ototoxic effects. To address this issue, we have begun to develop a standard screen for ototoxicity that can be used in drug development and drug safety analyses.

The zebrafish (*Danio rerio*) is increasingly recognized as a powerful model system for studying disease and for in vivo drug discovery. For example, the zebrafish has been used to identify compounds that can correct genetic heart defects (Peterson and Fishman 2004), suppress cancer genes (Stern et al. 2005), and promote hematopoiesis (North et al. 2007). The lateral line organ of zebrafish demonstrates unique advantages that make it useful for investigating hair cell toxicity. The hair cells of the lateral line reside in groups called neuromasts that are located in stereotyped positions on the surface of the head and body, making the hair cells easily accessible for exposure to chemicals (Fig. 1). The hair cells share both morphological and functional similarity to those of the mammalian inner ear. In addition, the zebrafish larva is optically transparent, and the hair cells of the lateral line readily take up fluorescent dyes, such as YO-PRO1. These two factors allow rapid examination of hair cells in vivo using fluorescence microscopy. Finally, the zebrafish has high fecundity, with usual clutch sizes greater than 100 in number (Hertog 2005). Our high throughput screening protocol takes advantage of these large numbers of animals by screening large numbers of chemicals in a relatively short amount of time using a single clutch of animals.

Aminoglycoside and cisplatin-induced hair cell death in the zebrafish lateral line has been studied in detail (Harris et al. 2003; Murakami et al. 2003; Ton and Parng 2005; Santos et al. 2006; Owens et al. 2007a; Ou et al. 2007). All of these studies have helped to validate the use of zebrafish as a screening tool for ototoxicity, but they have focused largely on the realm of known ototoxic agents. This study focuses on the detection of unknown ototoxic agents from a large library of compounds. We used the zebrafish lateral line to screen a library of 1,040 FDA approved compounds and bioactives for ototoxic effects. Twenty-one compounds were identified as selectively toxic to zebrafish hair cells. Dose–response relationships were examined for all 21 compounds. As proof of concept that these findings may be applicable to mammals, the ototoxic effects of two drugs identified in the zebrafish screen were confirmed in mature mouse utricular explants.

MATERIALS AND METHODS

Animals

Zebrafish

Zebrafish embryos were obtained from matings between AB wildtype zebrafish at the University of Washington fish facility. At 4 days postfertilization (dpf), larvae were

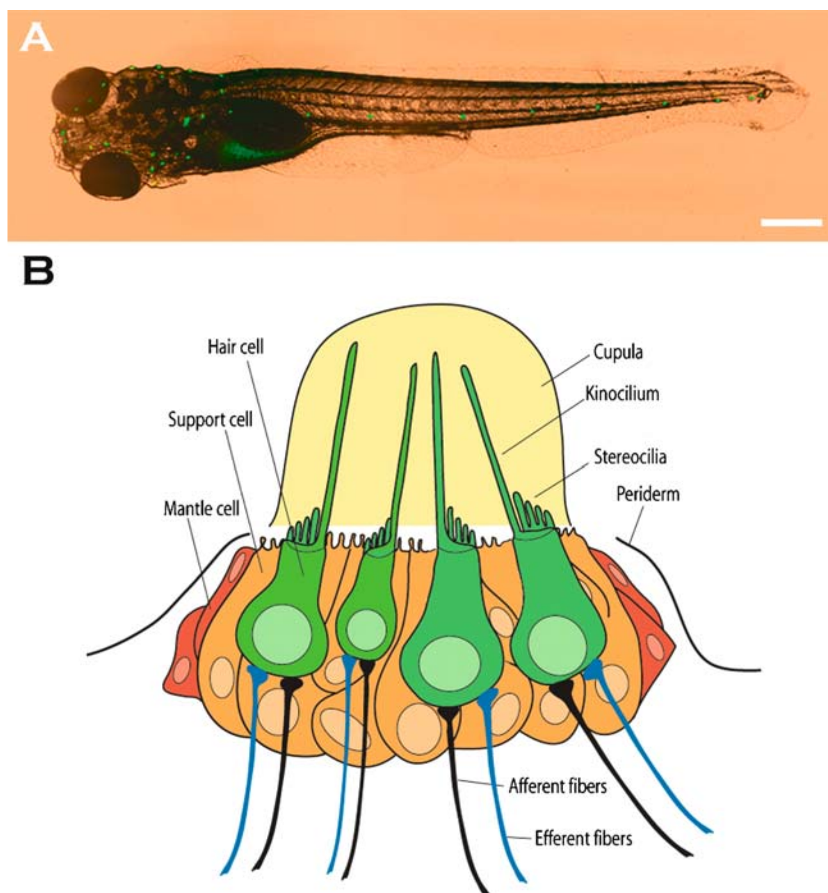

FIG. 1. **A** Live preparation of fluorescently labeled zebrafish larva 5 dpf (Harris et al. 2003). Neuromasts of the lateral line are stained with YO-PRO1. *Scale bar*=0.5 mm. **B** Schematic illustration of a neuromast. Hair cells are depicted in *green* with long kinocilia and shorter stereocilia projecting from the apical end of the cells and afferent and efferent nerve fibers at the basal end. Support cells (*orange cells*) intercalate between the hair cells.

fed live paramecia and plant-based food. Larvae were maintained in fish embryo media (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 in a tissue culture incubator at 28.5°C. All zebrafish protocols were approved by the University of Washington Institutional Animal Care and Use Committee.

Mice

Four- to 6-week-old CBA/J mice were obtained from Harlan Sprague Dawley (Harlan) and maintained in either the University of Washington Animal Care Facility or the Medical University of South Carolina Animal Care Facility. All mouse protocols were approved by both the University of Washington and the Medical University of South Carolina Institutional Animal Care and Use Committees.

Drug library

A commercial library of 1,040 FDA-approved drugs and bioactives, the NINDS Custom Collection II (Microsource Discovery Systems) was screened. The library consisted of 13 microplates of 80 drugs each. The scientist was blinded to the identities of the individual compounds. The full list of compounds is available from Microsource (www.msdiscovery.com)

Initial screen in zebrafish

Approximately 100 5-dpf fish larvae were prepared for use with drug compounds from each of 13 commercial drug stock microplates. Hair cells of the lateral line were labeled with YO-PRO1 (Invitrogen), a cyanine monomer vital fluorescent dye that can localize specifically to hair cell deoxyribonucleic acid (DNA; Santos et al. 2006). Fish larvae were exposed to 2 μM YO-PRO1 in embryo media for 30 min and washed with embryo media four times. Fish were placed (one fish per well) into a Nunc 96-well optical bottom plate (Thermo Fisher Scientific). Drugs from the library were then added (one drug per well) to each well using a multichannel micropipette for a final concentration of 100 μM. Fish were incubated in the drug for 1 h and then anesthetized with MS-222 (3-aminobenzoic acid

ethyl ester, methanesulfonate salt, Sigma). Fluorescence microscopy, using an automated microscopy stage, allowed for rapid imaging of the 96-well plate (Marianas imaging system, Intelligent Imaging Innovations) utilizing a Zeiss Axiovert 200M inverted microscope (Carl Zeiss). Fish viability was confirmed under microscopy by visualization of heartbeat and blood flow. Hair cells within the neuromasts of the lateral line were observed for morphological signs of injury (primarily nuclear condensation and fragmentation). Nuclear enlargement was rarely noted. Each fish was assigned a grade according to a pre-established grading system of 0–4 (Fig. 2), 0 being the negative control (no treatment, 1% dimethylsulfoxide only) and 4 being the positive control (complete or nearly complete loss of hair cells, neomycin 200 μM). Eight negative control wells and eight positive control wells were included on each 96-well plate, distributed evenly with one positive control and one negative control per plate row. Duration of screening time for each 96-well plate was 30 to 45 min.

Confirmatory retest in zebrafish

Drugs assigned a grade between 1 and 4 in the initial screen were regarded as being potentially ototoxic. These potentially ototoxic drugs were rescreened in triplicate in a confirmatory retest. Each drug was retested on three fish distributed into three separate wells, one fish per well. Conditions were identical to those of the initial screen protocol with a drug incubation time of 1 h and concentration of 100 μM. Visualization and grading of hair cell morphology was achieved within 10 min from the endpoint time of drug incubation.

Dose–response curves in zebrafish

Dose–response relationships were studied in the zebrafish lateral line for all 21 compounds identified in the confirmatory retest. Ten to 15 fish were tested at each of the following concentrations of each drug: 0, 50, 100, 200, and 400 μM. To assess hair cell survival, we used FM1-43FX (Invitrogen), a fixable styryl pyridinium dye that labels hair cells of the lateral line and allows rapid assessment of hair cell survival in fixed tissue. Fish larvae were exposed to 4 μM FM1-43FX for 30 s, rinsed, and then exposed to drug compound at the described concentrations for 1 h. Fish were then removed from incubation and fixed with 4% paraformaldehyde for 2 h at room temperature, washed in phosphate-buffered saline (PBS), and mounted onto slides using Fluoromount-G (Southern Biotechnology). Lateral line hair cells of neuromasts SO1, SO2, O1, and OC1 (Raible and Kruse 2000) were visualized under fluorescence microscopy, and surviving hair cells were counted for each neuromast.

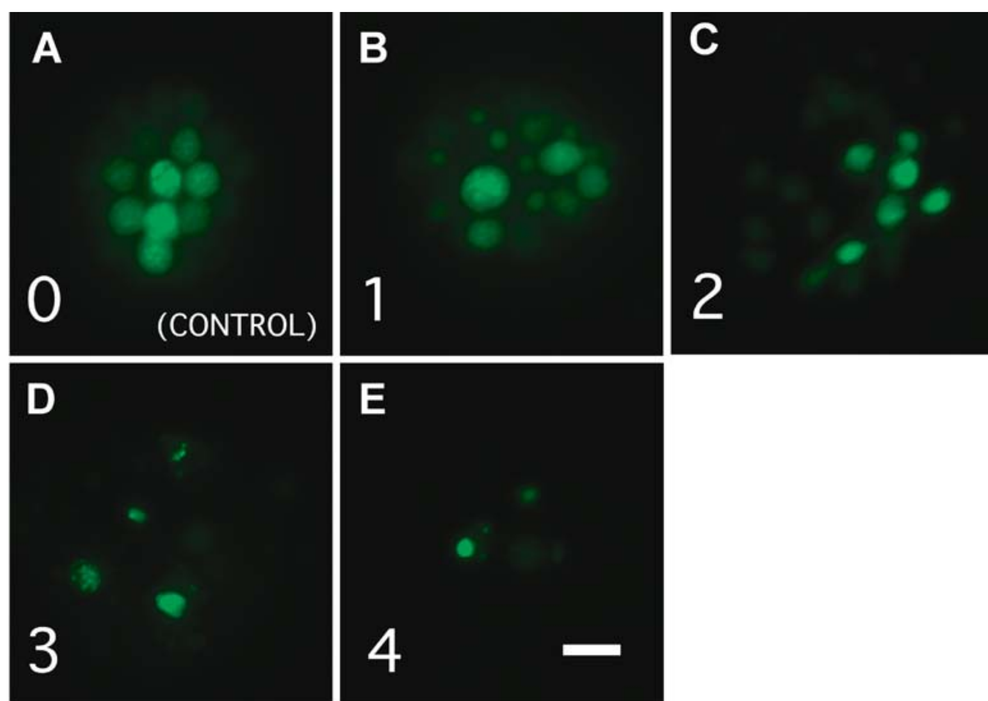

FIG. 2. **A**–**E** Examples of hair cell damage along with the grading system used for the initial screen. Each panel shows a single neuromast stained with YO-PRO1. Neuromasts range from undamaged control (**A**, grade 0), to subtle disorganization of hair cells and nuclear condensation (**B**, grade 1), to near complete hair cell loss (**E**, grade 4). *Scale bar* in **E**=10 μm and applies to all five panels.

Total numbers of surviving hair cells were compared to hair cell numbers from control animals from the same experiment (0 μM group).

Dose–response curves in mouse utricles

Two of the 21 compounds identified in the confirmatory retest were also tested in mouse utricle explant cultures. Utricles were isolated from 4- to 6-week-old CBA/J mice as described by Cunningham (2006) with the modification that the utricle was approached directly through the cranial side of the temporal bone. Careful attention was given to maintaining the integrity of the associated otoconia. The utricles were placed in untreated culture media (basal medium Eagle and Earle's balanced salt solution, 2:1 *v/v*, supplemented with 5% fetal bovine serum) and transferred into culture media with experimental doses of the respective drugs. Specific experimental doses of each compound were determined in preliminary studies, with the aim to optimally observe the comprehensive dose–response range. The utricles were cultured free-floating in sterile 24-well tissue culture plates (four to eight utricles per well) and incubated at 37° C in a 5% CO_2/95% air environment.

Immunocytohistochemistry for mouse utricle cultures

After 24 h, the mouse utricles were removed from incubation and otoconia carefully removed under a direct stream of PBS from a syringe with a 27-G needle. Utricles were then fixed at room temperature for 2 h in 4% paraformaldehyde with gentle shaking. After fixation, utricles underwent washes in PBS, followed by a 3-h exposure in blocking solution (PBS supplemented with 2% bovine serum albumin, 0.4% normal goat serum, 0.4% normal horse serum, and 0.4% Triton X-100). The blocking solution was removed, and the utricles were incubated overnight in primary antibody diluted in blocking solution (calmodulin 1:200, Sigma, calbindin 1:250, Chemicon). Calmodulin labels all hair cells of the utricle, and calbindin specifically labels hair cells in the striolar region. After washing thoroughly in PBS and 0.1% Triton X-100, the utricles were incubated with secondary antibodies in blocking solution for 2 h at room temperature (1:400 Alexa-488 goat anti-mouse IgG, 1:400 Alexa 594 goat anti-rabbit IgG, Invitrogen). In some cases, bisbenzimide (Invitrogen), used as a fluorescent DNA marker, was added for the second hour of incubation (10 μg/ml).

Mouse utricle hair cell counts

Using fluorescence microscopy, calbindin-labeled (red) hair cells of the striolar region were counted in each of four randomly designated 900-μm^2 areas using a Texas Red filter set. Calmodulin-labeled (green) hair cells in the extrastriolar region were counted in each of four randomly selected 900-μm^2 areas using a fluorescein isothiocyanate (FITC) filter set. Overall tissue viability was confirmed using bisbenzimide, a fluorescent DNA label that allowed visualization of surrounding support cells, as well as hair cells, under a diamidinophenylindole (DAPI) filter set. The four striolar hair cell counts and four extrastriolar hair cell counts were each, respectively, averaged to produce one striolar and one extrastriolar hair cell density for each utricle examined. Five to eight utricles were examined for each experimental condition.

Statistics

All values were calculated and presented as the mean value±one standard deviation. Statistical analyses were performed using one-way analysis of variance (ANOVA; Vassarstats, http://faculty.vassar.edu/lowry/VassarStats.html). Results were considered statistically significant if $p<0.05$.

RESULTS

The initial screen has high sensitivity

Of the 1,040 compounds included in the NINDS Custom Collection II library, the initial screen detected 95 compounds to be potentially ototoxic (i.e., having a grade of 1–4). This group of candidate ototoxins represented approximately 9% of the entire library. The candidate group consisted of compounds from a wide variety of drug classes and origins. For these candidate ototoxins, the overall health of the fish itself was confirmed with visualization of heartbeat and blood flow.

Confirmatory retest improves specificity

Of the 95 potentially ototoxic drugs from the initial screen, 21 were confirmed by triplicate retest to be injurious to hair cells of the zebrafish lateral line, having an average grade of 1–4 (Table 1). These 21 compounds represent 2% of the entire NINDS Custom Collection II library and roughly 22% of the initial candidate group, for a false positive rate of 78%. The retest values were averaged for each compound and are shown in Table 1. Several drug classes were represented, including aminoglycoside antibacterial agents: tobramycin, neomycin, and kanamycin, other antibacterial agents: chlortetracycline, chloramphenicol, and demeclocycline, antiprotozoal agents: pentamidine and mefloquine, an anti-

TABLE 1

Candidate ototoxic compounds detected by the screening protocol

Grade	*Compound*	*Class*	*Known ototoxicity?*
4	Chloramphenicol	Antibiotic	Rare case reports
4	Chlortetracycline HCl	Antibiotic	No
4	Pentamidine isethionate	Antiprotozoal	No
3.3	Spermadine	Ornithine decarboxylase inhibitor	No
3	Tobramycin	Antibiotic	Yes
3	Propantheline bromide	Anticholinergic	No
3	Ethacrynic acid	Loop diuretic	Yes
2.7	Pomiferin	Antioxidant	No
2.7	Chlorophyllide	Antineoplastic, chlorophyll derivative	No
2.3	Estradiol valerate	Estrogen	Rare case reports
2.3	Neomycin	Antibiotic	Yes
2.3	Pentetrazole	CNS/respiratory/circulatory stimulant	Yes, animal studies
2	Guaiazulene	Antioxidant, color additive agent	No
1.3	Rosolic acid	Diagnostic aid	No
1	Cisplatin	Antineoplastic	Yes
1	Vincamine	Vasodilator	No
1	Kanamycin	Antibiotic	Yes
1	Demeclocycline HCL	Antibiotic	No
1	Mefloquine	Antiprotozoal	Yes
1	Candesartan	Angiotensin 1 receptor antagonist	No
1	Simvastatin	HMGCoA reductase inhib., antihyperlipidemic	No
Known ototoxic drugs not detected in screen of NINDS Custom Collection II library			
0	Amikacin	Antibiotic	Yes
0	Gentamicin	Antibiotic	Yes
0	Furosemide	Loop diuretic	Yes
0	Carboplatin	Antineoplastic	Yes
0	Quinine	Antiprotozoal	Yes

Compounds are listed in order of decreasing mean injury grade as determined by confirmation retest and are accompanied by respective drug class and known ototoxicity status. The second group of drug compounds shown below are known ototoxic drugs included in the library of drugs used in this study not detected by the screening protocol.

neoplastic agent: cisplatin, an anticholinergic agent: propantheline bromide, and an antihyperlipidemic agent: simvastatin. Seven of the 21 compounds on the list have known ototoxicity, either commonly known or found in its respective drug profile list of possible adverse events: tobramycin, ethacrynic acid, neomycin, cisplatin, kanamycin, mefloquine, and arguably chloramphenicol (Roland and Rutka 2004). Some known ototoxic drugs did not demonstrate hair cell injury based on the initial screening protocol used in this study. These drugs were gentamicin, amikacin, furosemide, carboplatin, and quinine (Table 1).

Dose–response relationships in hair cells of the zebrafish lateral line

The relationship between drug concentration and hair cell survival was investigated in the lateral line neuromasts for each confirmed drug compound (Table 2). The average total number of hair cells per fish was determined from the four representative neuromasts for each drug concentration condition. Hair cell total was expressed as the percentage of surviving hair cells relative to untreated control conditions. This assessment controlled for inherent variability of hair cell number from one group of fish to another. All compounds with the exception of cisplatin, demeclocycline, and pentetrazole demonstrated statistically significant dose–dependent hair cell loss ($p<0.05$, one-way ANOVA). It is noteworthy that more extensive investigations with a range of exposure duration and concentration with cisplatin have shown a consistent, predictable dose–response relationship (Ou et al. 2007). Similar findings may be observed with demeclocycline and pentetrazole when more extensive testing is completed. Figure 3A and B show examples of dose–response functions for pentamidine isethionate and propantheline bromide, two compounds from the confirmed candidate list.

Hair cell injury and dose–response relationships are conserved in hair cells of mouse utricle explants

Dose–response relationships were studied in mature mouse utricle explant cultures treated with pentami-

TABLE 2

Dose–response relationships of candidate ototoxic drugs represented as hair cell survival after treatment with increasing doses of each drug

	Hair cell survival (% of control)					
Drug	*0 μM*	*50 μM*	*100 μM*	*200 μM*	*400 μM*	*ANOVA*
Candesartan	100±11	90±9	82±9	67±13	Dead	$p<0.0001$
Chloramphenicol	100±15	42±18	19±7	19±10	10±11	$p<0.0001$
Chlorophyllide	100±18	92±7	47±8	24±15	7±6	$p<0.0001$
Chlortetracycline	100±15	101±10	79±24	63±21	43±16	$p<0.0001$
Cisplatin	100±12	93±12	94±10	95±12	88±13	$p=0.22$
Demeclocycline	100±12	101±14	98±13	92±8	93±9	$p=0.21$
Estradiol valerate	100±16	60±16	56±14	46±16	Dead	$p<0.0001$
Ethacrynic acid	100±8	70±17	52±17	44±12	Dead	$p<0.0001$
Guaiazulene	100±11	83±13	54±9	52±15	Dead	$p<0.0001$
Kanamycin	100±17	98±14	86±15	84±10	85±6	$p<0.05$
Mefloquine	100±13	91±14	81±19	76±17	Dead	$p<0.01$
Neomycin	100±14	74±8	43±9	18±4	4±4	$p<0.0001$
Pentamidine	100±6	68±9	57±13	54±11	36±10	$p<0.0001$
Pentetrazole	100±13	99±14	91±8	89±9	92±14	$p=0.14$
Pomiferin	100±15	44±18	45±18	45±18	Dead	$p<0.0001$
Propantheline	100±12	68±16	53±16	49±10	18±8	$p<0.0001$
Rosolic acid	100±12	83±16	68±14	22±11	23±9	$p<0.0001$
Simvastatin	100±9	67±13	48±12	Dead	Dead	$P<0.0001$
Spermadine	100±16	61±10	7±6	1±2	1±1	$p<0.0001$
Tobramycin	100±12	95±17	53±15	49±10	33±15	$P<0.0001$
Vincamine	100±12	69±14	47±15	45±16	13±8	$p<0.0001$

Dose dependency was evaluated with one-way ANOVA. Some drug doses were toxic to the fish and are indicated as "Dead."

dine or propantheline. These two drugs were selected due to their potent toxicity to hair cells without overall toxicity to the zebrafish. Figure 4 shows pentamidine-treated utricles double-labeled with antibodies to calmodulin and calbindin. Following 24-h culture, the untreated control utricle appeared viable and in good condition, with uniform hair cell density and quality throughout both the striolar and extrastriolar regions (Fig. 4A). In comparison, mouse utricles treated with pentamidine exhibited signs of hair cell injury as well as decreased hair cell numbers at all of the doses examined (Fig. 4B–F). In separate experiments, overall tissue viability was confirmed by visualization of bisbenzimide-labeled support cells, confirming that the tested compounds appeared to demonstrate selective hair cell toxicity. Figure 5 shows the dose–response relationship for mouse utricle hair cells treated with pentamidine. Hair cell density is expressed as a percentage of surviving hair cells relative to untreated control utricles. Hair cell density decreased as the pentamidine concentration increased for both the striolar and extrastriolar regions until no hair cells remained in the utricles cultured in 200 μM pentamidine. In both the striolar and extrastriolar regions, hair cell density decreased in a dose-dependent fashion compared to the untreated control utricles ($p<0.0001$, one-way ANOVA).

Propantheline-treated utricles also demonstrated extensive hair cell loss on fluorescence microscopy (Fig. 6). Striolar hair cells were less sensitive than extrastriolar hair cells to the damaging effects of propantheline, but both extrastriolar and striolar hair cells demonstrated a significant ($p<.0001$, one-way ANOVA) dose-dependent decrease in hair cell density (Fig. 7).

DISCUSSION

We have described the development and validation of a method for screening and then evaluating drugs for potential ototoxicity. In this study, a library of 1,040 FDA-approved drugs and bioactive compounds was rapidly screened in zebrafish lateral line hair cells for hair cell toxicity. This library was selected because it was one of the largest commercially available collections of FDA-approved drugs, and thus any findings would be more clinically relevant. The screening methodology generated a list of potential candidate ototoxins, and dose–response relationships were further assessed in lateral line neuromasts. For validation of the screen, 2 of the 14 identified potential ototoxins were then tested in mature mouse utricle explants in vitro. These two compounds that were first identified as potentially ototoxic from the zebrafish lateral line testing were also shown to be toxic to mammalian hair cells using the mouse utricle preparation. Demonstration of conserved injurious effects in the

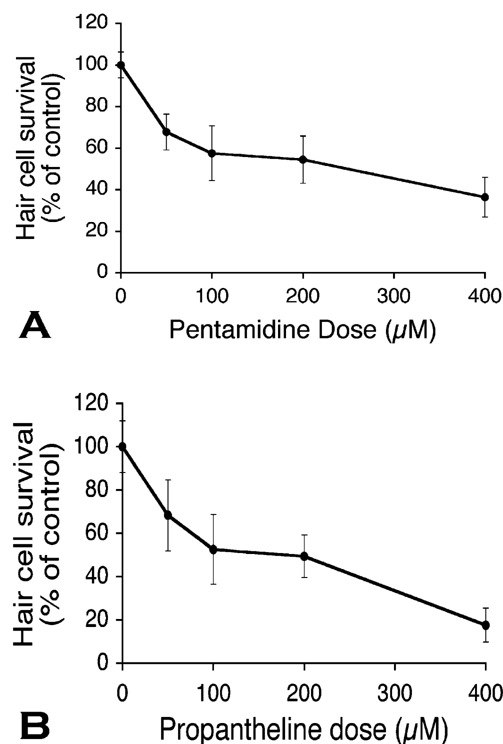

FIG. 3. Dose–response relationships of pentamidine and propantheline-exposed zebrafish. Zebrafish larvae at 5 dpf were exposed to various concentrations of pentamidine isethionate (**A**) or propantheline bromide (**B**) and were evaluated 1 h after exposure for surviving hair cells in neuromasts SO1, SO2, O1, and OC1 (n=10–15 larvae per condition). *Data points* represent the percentage of hair cell survival compared to the control (no drug) condition (±1 SD). Hair cell survival decreased with increasing concentrations of pentamidine ($p<0.0001$, one-way ANOVA) and propantheline ($p<0.0001$, one-way ANOVA).

mammalian cultures is an important step toward validating the zebrafish lateral line system as a screening tool for ototoxicity.

To our knowledge, this represents the first attempt for medium- or high-throughput screening of chemicals for ototoxicity. Currently, there is no required test for ototoxicity in drug evaluation protocols. As a result, there are likely many drugs currently in use that have occult ototoxic effects. Similarly, new drugs are completing clinical trials and reaching the public with no knowledge of ototoxic potential.

Alternative approaches to screening

For obvious reasons, high- or even medium-throughput in vivo screens in larger animal models are not feasible. Similarly, it is unlikely that screens utilizing in vitro preparations of utricles or cochleae from larger animals will be routinely adopted, as thousands of animals would be required to screen even medium-sized compound libraries. Hair cell lines (Rivolta et al. 1998; Lawlor et al. 1999) provide an alternative model that is more compatible for screening; however, to date, no screen of ototoxicity in the few lines available has been reported. Furthermore, the use of hair cell lines is controversial because these cells have been selected to survive and thus may not be an ideal model to screen for hair cell death. The use of an in vivo screen has the additional benefit of addressing the complex metabolism of the live animal that influences actual drug efficacy and toxicity.

False positives and false negatives

Our protocol is a screening method, and inherent in any screen are both false positives and false negatives. Fortunately, in this screen, false positives were not a significant problem since the compounds were easily retested, and all false positives were quickly eliminated. False negatives were also expected since the goal of this study was not necessarily to detect all ototoxins in the library but rather to give insight into as many as possible and validate the method. The sensitivity and specificity of this screening protocol can be varied by varying the concentrations of the compounds used for screening and the length of time the fish is exposed to each compound. The current screen employed a standard concentration of 100 μM and a standard drug exposure time of 1 h. This standard dose of 100 μM was selected because it was close to a dose of neomycin that was known to be injurious to hair cells (Harris et al. 2003). This allowed us to identify and confirm 21 candidate ototoxins.

In the present study, 7 of 12 known ototoxins were successfully identified by the screen. However, there were also five clinically known ototoxins that were not identified in our screen (Table 1). Increasing the standard screening dose or duration would improve the sensitivity but also increase the false positive rate. Gentamicin was one of the drugs not identified as ototoxic in the present screen, which is consistent with data from our group showing that in zebrafish lateral line hair cells, gentamicin requires approximately a 3–6-h exposure for hair cell injury to occur at this concentration (Owens et al. 2007b). Likewise, cisplatin was identified by the screen but demonstrated minimal dose dependency (p=0.22, one-way ANOVA) at the exposure conditions tested (50 to 400 μM concentration for 1 h). These data are also consistent with published results showing that cisplatin requires exposure durations in the 4-h range to consistently damage lateral line hair cells (Ou et al. 2007). The commonly used loop diuretic, furosemide, was also not identified by the screen. This is also not surprising, since ototoxic effects of loop diuretics are thought to occur at the level of the stria vascularis. The lateral line system has no anatomic or physiologic equivalent of the stria and thus would not be expected to demonstrate toxicity

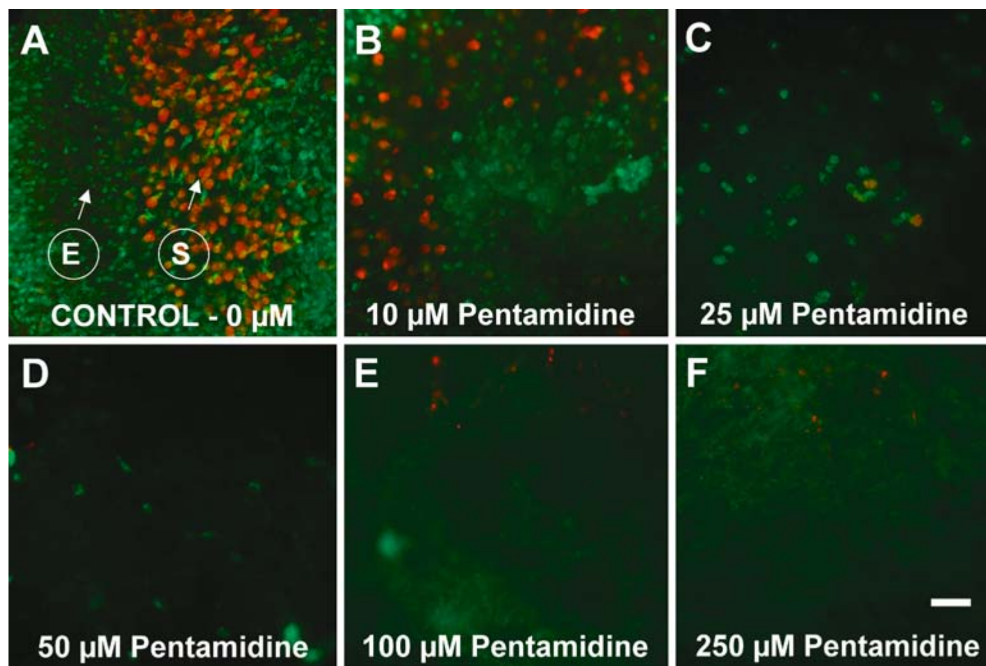

FIG. 4. Cultured mouse utricles treated with pentamidine and labeled for calmodulin and calbindin. Utricles were cultured for 24 h without pentamidine (control; **A**), or with pentamidine at 10 (**B**), 25 (**C**), 50 (**D**), 100 (**E**), and 250 μM (**F**). Following culture, utricles were fixed and double-labeled with antibodies directed against calmodulin (*green*) that label all hair cells of the utricle and calbindin (*red*) that label hair cells of the striolar region only. *S* indicates striolar hair cells, while *E* indicates extrastriolar hair cells. Treatment with 10 μM pentamidine resulted in a significant reduction in hair cell density as well as a swollen, damaged appearance of the hair cells. Treatment with increasing doses results in progressive injury up to 100- to 250-μM levels when all hair cells are absent. *Scale bar* in **F**=30 μm and applies to all panels.

through a strial mechanism. On the other hand, ethacrynic acid, another loop diuretic, appeared to be directly damaging to lateral line hair cells.

The fact that three compounds (cisplatin, demeclocycline, and pentetrazole) were detected on the screen but did not demonstrate dose dependence when more thoroughly tested speaks of the sensitivity of the initial screening method. It is important to note that initial screening utilized YO-PRO1 DNA staining, which allows easy assessment of relatively subtle nuclear changes that can occur prior to hair cell death. However, YO-PRO1 is not effectively fixable, making it difficult to use this dye for hair cell counts. On the other hand, fixable FM1-43 was used to assess dose–response relationships because it is effectively used for hair cell counts; it is efficiently used to detect the presence or absence of hair cells but cannot be used to assess more subtle nuclear changes. As a result, the initial screen using YO-PRO1 staining may be more sensitive to mild ototoxic effects. Since two of these three compounds (cisplatin and pentetrazole) have known ototoxicity, it is likely that if exposure parameters (dose and concentration) were altered for these drugs, a dose-dependent toxicity profile would become apparent (Ou et al. 2007).

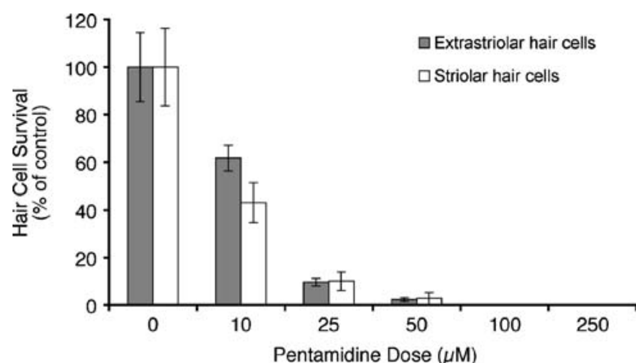

FIG. 5. Dose–response relationship of utricles treated with pentamidine. The density of hair cells of each utricle were quantified and averaged from four striolar and four extrastriolar representative regions of 900 μm^2 each. *Bars* represent the mean hair cell survival (% control) ± 1 SD. Hair cell survival decreases significantly as pentamidine doses increases ($p<0.001$, one-way ANOVA). Both striolar and extrastriolar regions are sensitive to pentamidine-induced injury (N=6–8 per condition).

Toxicity in zebrafish hair cells was confirmed in the mouse utricle

Two of the 14 novel ototoxic compounds, pentamidine and propantheline, were tested in mammalian utricles. These two drugs were chosen as they demonstrated potent hair cell toxicity, without toxicity to the zebrafish at higher doses. Only two drugs were tested for this

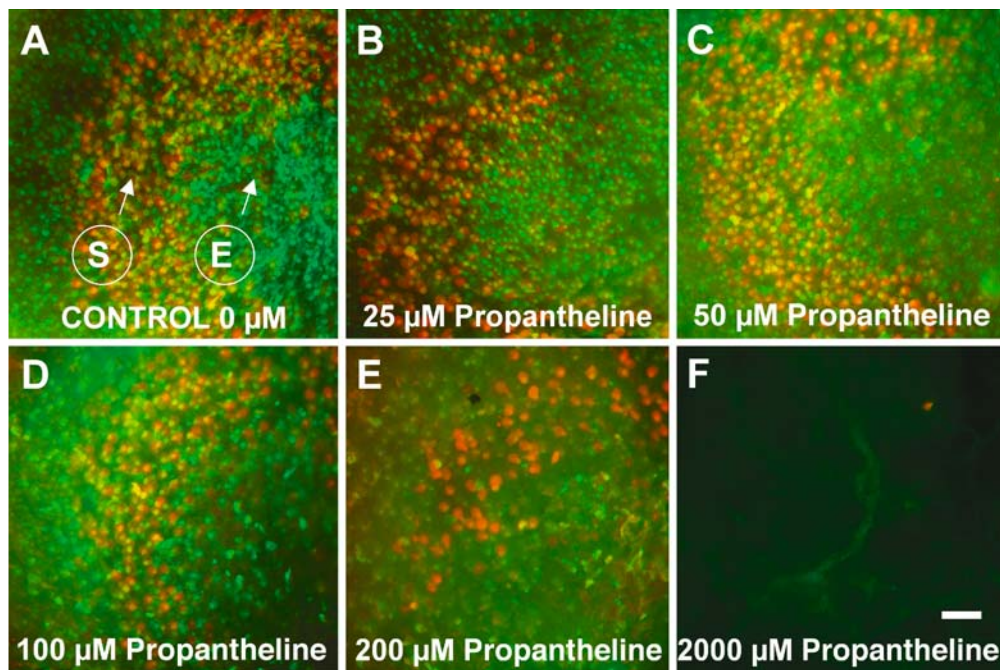

FIG. 6. Cultured mouse utricles treated with propantheline and labeled for calmodulin and calbindin. Utricles were cultured for 24 h without propantheline (control; **A**) or with propantheline at 25 (**B**), 50 (**C**), 100 (**D**), 200 (**E**), and 2,000 μM (**F**). *S* indicates striolar hair cells, while *E* indicates extrastriolar hair cells. Treatment with increasing doses of propantheline resulted in progressively higher degrees of hair cell loss. *Scale bar* in **F**=30 μm and applies to all panels.

report due to the time-consuming nature of mammalian testing. Our goal was to assess whether any drug identified by this screen showed inner ear toxicity as opposed to carrying out exhaustive mammalian inner ear evaluations.

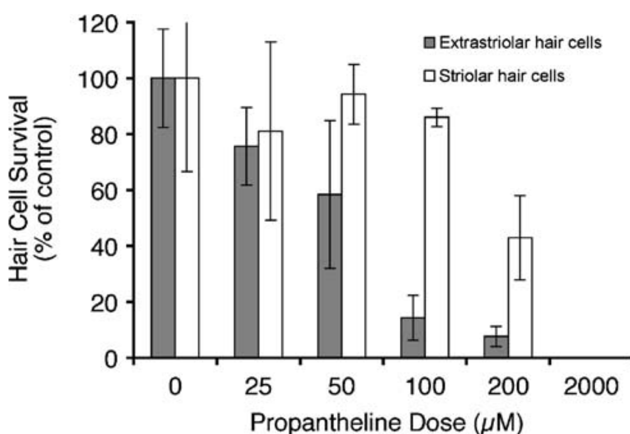

FIG. 7. Dose–response relationship of utricles treated with propantheline. Hair cells of each utricle were quantified and averaged from four striolar and four extrastriolar representative regions of 900 μm^2 each. *Bars* represent the mean hair cell survival (% control) ± 1 SD (N=3–8 per condition). Both extrastriolar and striolar hair cell survival decreased in a dose-dependent manner (p<0.0001, one-way ANOVA). The striolar region exhibited less hair cell loss than the extrastriolar region.

The clinical uses of these drugs deserve mention. While at one time proposed as a first-line therapy for *Pneumocystis carinii* pneumonia (PCP) in human immunodeficiency virus (HIV) patients and other immunocompromised individuals, pentamidine is now considered an alternative-line treatment. It is currently used as a first-line agent in patients with sensitivity to trimethoprim/sulfamethoxazole (TMP/SMX). Studies have indicated that pentamidine is either similar in effectiveness (intravenous route) or less effective (aerosolized route) than TMP/SMX against PCP; however, pentamidine is better tolerated with fewer reported adverse events (Schneider et al. 1992; May et al. 1994; Nielsen et al. 1995; Bellamy 2006). Pentamidine has also been studied as first-line treatment for other protozoal infections such as Old World cutaneous leishmaniasis (Hellier et al. 2000; Nacher et al. 2001; Lai A Fat et al. 2002; Roussel et al. 2006) and disseminated cutaneous leishmaniasis during HIV infection (Calza et al. 2001). Because pentamidine isethionate is commonly used in the HIV population, it becomes difficult to discriminate between hearing loss that is secondary to disease progression versus hearing loss that is secondary to an occult ototoxic effect of disease treatment. Studies in a South African population with HIV showed an increase in the occurrence of sensorineural hearing loss with the decline of patients' immunological status, with patient history data suggesting that both

opportunistic infections and their treatments were contributing causes (Hausler et al. 1991; Khoza and Ross 2002; Reyes-Contreras et al. 2002).

Propantheline bromide is another potential ototoxin identified in this screen and subsequently tested in the mouse utricle model. It is an anticholinergic agent used in the setting of hyperhidrosis or excessive sweating, gastrointestinal ulcer disease, neurogenic bladder disease, urinary incontinence, or irritable bowel syndrome. Propantheline treats medical diagnoses more typically seen in the aging population, and thus any ototoxic effect may be masked by presbycusis. Similarly, simvastatin, a very commonly used antihyperlipidemic agent, and candesartan, a commonly used antihypertensive agent, were both identified in our study as being potentially ototoxic. Similar to propantheline, both agents are most frequently used in the aging population and hence could have ototoxic effects masked by presbycusis.

Striolar versus extrastriolar hair cell loss

The pattern of hair cell loss seen in pentamidine- and propantheline-exposed utricles differs from what has been seen in aminoglycoside-exposed utricles. Aminoglycosides have been shown to cause more striolar than extrastriolar hair cell loss (Cunningham et al. 2002), which may be due to the increased prevalence of type 1 hair cells within the striola. In contrast, propantheline caused primarily extrastriolar hair cell loss, while pentamidine showed roughly equivalent striolar and extrastriolar loss. These findings suggest differences in the mechanisms of uptake of propantheline and pentamidine into type 1 versus type 2 hair cells or differences in the death pathways initiated once uptake has occurred.

Some drugs may have both protective and toxic effects

It is interesting to note that two of the compounds identified in the study as being potentially ototoxic with respect to hair cells have been suggested in the literature to be protective against hearing loss through mechanisms other than their actions on hair cells. The statin family of drugs, HMG CoA-reductase inhibitors, was once hypothesized to be useful as a treatment for sensorineural hearing loss through their metabolic and hemodynamic effects (Borghi et al. 2002). Studies with atorvastatin-treated mice have shown decreased expression of intercellular and vascular adhesion molecules in the aortic wall, suggesting that reducing endothelial inflammatory effects may contribute to improved hair cell function by influencing the blood supply to the inner ear (Syka et al. 2007). A similar paradox was found for estrogens, which have been suggested by many to have favorable vascular effects that are protective of hearing (Kilicdag et al. 2004; Hultcrantz et al. 2006); estradiol valerate was identified in our study as being ototoxic to hair cells of the zebrafish lateral line. Whether any protective effects of simvastatin or estrogen will exceed the potential ototoxic effects on hair cells is unknown, and testing in vivo in a mammalian model is needed to provide further insight into this question. It is known that some compounds such as Jun kinase inhibitors can have protective effects at low doses but toxic effects at higher doses (Ou et al. 2006; Sugahara et al. 2006).

Caveats

The zebrafish lateral line represents a model system for screening a large number of chemicals for potentially ototoxic effects. It is important to note that since the lateral line lacks a stria vascularis, this screen will not identify drugs that cause hair cell loss through strial mechanisms. In addition, lateral line hair cells resemble vestibular hair cells more than cochlear hair cells, and hence, we could miss drugs that are highly selective for auditory hair cells and relatively benign with respect to vestibular hair cells. Furthermore, fish are not mammals, and thus all findings require confirmation in mammalian systems, preferably in vivo. In this study, the ototoxic effects were confirmed in both of the drugs we tested in the mouse utricle. In vivo mammalian studies are critical as the inner ear penetrance of any of these potential ototoxins is largely unknown. These tests will await future investigations by our group and others.

CONCLUSIONS

We have used the zebrafish lateral line to rapidly screen a large library of drugs for ototoxic effects. It represents a unique and powerful tool for studying hair cells. Our findings suggest that there may be FDA-approved compounds in current clinical use with occult ototoxic effects. This overall screening approach is easily adapted to applications with other groups of compounds, including the remaining currently FDA-approved drugs not in the NINDS Custom Collection II library (of which there are greater than 10,000), newly discovered drugs, and environmental chemicals.

There is no current standard screen for ototoxicity in drug development. As subtle hearing loss is easily missed, particularly in children or the elderly, we feel that it is critical to develop a tool to identify drugs with potential ototoxicity. Pre- and post-treatment audiograms during clinical trials would then be recommended for any drug found to be a potential ototoxicant. While some have criticized the number of hurdles a drug must face to achieve FDA approval, we feel that

there is an ethical obligation to identify drugs that are potentially damaging to hearing.

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