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Research paper

Screen of FDA-approved drug library reveals compounds that protect hair cells from aminoglycosides and cisplatin

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ABSTRACT

Loss of mechanosensory hair cells in the inner ear accounts for many hearing loss and balance disorders. Several beneficial pharmaceutical drugs cause hair cell death as a side effect. These include aminoglycoside antibiotics, such as neomycin, kanamycin and gentamicin, and several cancer chemotherapy drugs, such as cisplatin. Discovering new compounds that protect mammalian hair cells from toxic insults is experimentally difficult because of the inaccessibility of the inner ear. We used the zebrafish lateral line sensory system as an in vivo screening platform to survey a library of FDA-approved pharmaceuticals for compounds that protect hair cells from neomycin, gentamicin, kanamycin and cisplatin. Ten compounds were identified that provide protection from at least two of the four toxins. The resulting compounds fall into several drug classes, including serotonin and dopamine-modulating drugs, adrenergic receptor ligands, and estrogen receptor modulators. The protective compounds show different effects against the different toxins, supporting the idea that each toxin causes hair cell death by distinct, but partially overlapping, mechanisms. Furthermore, some compounds from the same drug classes had different protective properties, suggesting that they might not prevent hair cell death by their known target mechanisms. Some protective compounds blocked gentamicin uptake into hair cells, suggesting that they may block mechanotransduction or other routes of entry. The protective compounds identified in our screen will provide a starting point for studies in mammals as well as further research discovering the cellular signaling pathways that trigger hair cell death.

1. Introduction

There are no clinically approved and effective drugs that protect patients against the ototoxic side effects of important pharmaceutical agents such as aminoglycoside antibiotics and anti-neoplastic drugs (e.g. cisplatin and carboplatin). We seek co-treatments that diminish or eliminate the toxic side effects of these beneficial drugs. As access to mature mammalian auditory and vestibular tissue prevents large-scale screening for modulators of hair cell death, we have used the larval zebrafish lateral line system for screening (reviewed in Ou et al., 2010; Coffin et al., 2010). The zebrafish lateral line is a sensory system that contains mechanosensory hair cells in clusters called neuromasts located in stereotyped positions along the body and head of the larval and adult fish (Metcalfe et al., 1985; Raible and Kruse, 2000). Lateral line hair cells share structural, functional and molecular similarities to mammalian inner ear hair cells (reviewed in Coombs et al., 1989; Nicolson, 2005). Many zebrafish genes have been identified that function in hearing and balance with phenotypes similar to that of their mammalian counterparts including components of the hair cell transduction apparatus (Nicolson et al., 1998; Ernest et al., 2000). The hair cells in this system present several advantages for screening: they can be visualized in vivo because the hair cells are located on the outside of the body and readily take up vital dyes (Harris et al., 2003; Santos

Abbreviations: dpf, days post-fertilization; EM, embryo medium; GTTR, gentamicin-Texas Red; MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration; SERM, selective estrogen receptor modulator; SSRI, selective serotonin reuptake inhibitor.

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et al., 2006). Lateral line hair cells, like their mammalian counterparts, are sensitive to ototoxins, such as aminoglycosides, cisplatin and other chemotherapeutic drugs; and cell death can be reliably induced in a dose-dependent fashion (Williams and Holder, 2000; Harris et al., 2003; Ton and Parng, 2005; Ou et al., 2007; Owens et al., 2009; Hirose et al., 2011). Furthermore, zebrafish lateral line hair cells demonstrate morphological changes similar to the inner ear hair cells of birds and mammals when exposed to aminoglycosides (Owens et al., 2007) indicating that this is a robust model for understanding mammalian ototoxicity. The larvae are small, can be produced in large numbers, and are easy to handle, allowing addition to 96-well plates and rapid visualization of many individuals.

Aminoglycoside antibiotics, including gentamicin, kanamycin and neomycin, are antibacterial agents that are used worldwide for gram-negative bacterial infections. Depending on the country, they are used regularly or are reserved for use in more severe infections, e.g. tuberculosis. Aminoglyocosides kill bacteria by inhibiting ribosome function (Davis, 1987) and may lead to production of hydroxyl radicals that contribute to bacterial cell death (Kohanski et al., 2007). Besides their beneficial toxic effects against bacteria, aminoglycosides can cause nephrotoxicity as well as hearing loss and vestibular dysfunction due to hair cell death in humans (Hinshaw et al., 1946), rodents (Brummett, 1983), birds (Fermin et al., 1980), and fish (Kaus, 1992; Lombarte et al., 1993; Williams and Holder, 2000; Harris et al., 2003). Cisplatin is a valuable and widely used anti-cancer drug that disrupts cell division by creating DNA adducts (Rosenberg, 1985). Hearing loss and hair cell loss due to cisplatin exposure has been observed in humans (Reddel et al., 1982; Rosenberg, 1985), rodents (Fleischman et al., 1975) and fish (Ou et al., 2007).

We have screened drug and small molecule libraries for compounds that protect hair cells from neomycin toxicity (Ou et al., 2009; Owens et al., 2008). Those screens revealed several compounds with previously unknown protective properties and two compounds have proven effective in mammalian inner ear in vitro or in vivo (Owens et al., 2008; Ou et al., 2009; Rubel et al., 2011). Given this success, screening additional libraries of clinically approved drugs that might protect against a spectrum of hair cell toxins may be clinically useful and provide additional insights into the processes occurring in hair cells. Among the aminoglycosides, ototoxicity and tissue sensitivity differ (Dulon et al., 1986; Selimoglu et al., 2003; Smith et al., 1977; Wanamaker et al., 1999). Furthermore, aminoglycosides can exhibit divergent kinetics and potency. For example, in zebrafish neuromasts, gentamicin employs at least two processes leading to cell death: one short-term and another longer-term, while neomycin may activate only a short-term process (Owens et al., 2009). Cisplatin likely employs separate processes leading to cell death compared to aminoglycosides, and zebrafish mutations that protect against aminoglycosides do not protect against cisplatin (Owens et al., 2008). However, studies of hair cell ultrastructure suggest that mitochondria are early targets of both aminoglycosides and cisplatin (Owens et al., 2007; Giari et al., 2011). The possibility of a co-treatment effective with all aminoglycosides or with both aminoglycosides and cisplatin is enticing.

In the experiments presented below, we screened a library of FDA-approved drugs for compounds that protect hair cells of the zebrafish lateral line from the hair cell toxins neomycin, gentamicin, kanamycin and cisplatin. The Enzo Life Sciences FDA-approved drug library contains 640 compounds that have been or are currently used clinically. Of the 640 compounds in the library, ten compounds were found that protected hair cells treated with at least two of the four toxins. These compounds were subjected to further testing to examine the properties of their protective effects. The main classes of protective compounds were serotonin and dopamine-modulating drugs, adrenergic receptor ligands, and estrogen receptor modulators. Estrogen receptor modulators were further investigated and we identified three additional estrogen receptor modulators that protect hair cells from neomycin, though differences in their protective effects with gentamicin suggest that the compounds may be acting by multiple mechanisms.

2. Materials and methods

2.1. Animal care

Larval zebrafish (*Danio rerio*) of the *AB wildtype strain were produced via group matings of adult fish. Larvae were housed at 28.5 °C and maintained at a density of 50 fish per 10 cm diameter petri dish in embryo media (EM; 994 μM $MgSO_4$, 150 μM KH_2PO_4, 42 μM Na_2HPO_4, 986 μM $CaCl_2$, 503 μM KCl, 14.9 mM NaCl, and 714 μM $NaHCO_3$, pH 7.2). Beginning at 4 days post-fertilization (dpf), fish were fed live rotifers daily. Experiments were performed using 5–6 dpf larvae. The University of Washington Animal Care and Use Committee approved of the animal procedures described here.

2.2. Drug library

Enzo's FDA Approved Drug Library (Enzo Life Sciences Inc., Plymouth Meeting, PA, USA, formerly BIOMOL International, L.P.) was used to screen zebrafish larvae for compounds that protect against toxin-induced lateral line hair cell death. The library consists of 640 compounds dissolved at 2 mg/ml in dimethyl sulfoxide (DMSO; Sigma–Aldrich, #D1435). The compounds were aliquoted into eight 96-well plates with one compound per well and 80 compounds per plate. The plates were stored at 4 °C during initial screening and re-testing.

2.3. Screening

Larvae were pre-labeled with 2 μM YO-PRO1 (Invitrogen, Carlsbad, CA, USA; #Y3603) in embryo medium for 30 min and then rinsed three times. YO-PRO1 is a cyanine monomer fluorescent vital dye that labels hair cell DNA (Santos et al., 2006). After pre-labeling, larvae were transferred to Nunc 96-well optical bottom plates (Thermo Fisher Scientific, #265301), with one fish per well in 147 μL of embryo medium. Library compounds were diluted 1:10 in embryo medium and then 3 μL of the diluted mixture were added to 96 well plate containing larvae (one compound per well) for a final concentration of 4 μg/ml of library compound and final DMSO concentration of 0.2% in each well. Larvae were incubated for 1 h with library compounds, then one of the following hair cell toxins was added and fish were incubated in library compound and hair cell toxin together. The duration and concentration of the toxin was adjusted to kill most hair cells in each neuromast under control conditions without any of the library drugs but at the same DMSO concentration: 200 μM neomycin (Sigma, #N1142) for 1 h; 50 μM gentamicin (Sigma, #G1397) for 6 h; 400 μM kanamycin (Abraxis Pharmaceuticals Products) for 24 h; or 50 μM cisplatin (Bedford Laboratories) for 24 h (Harris et al., 2003; Ou et al., 2007; Owens et al., 2009). Eight fish in each plate (in column 1) served as mock controls and received no treatment (negative controls). Eight more fish (in column 12) were treated with hair cell toxin but no library drug to serve as positive controls for toxin potency. After incubation in library compound and hair cell toxin for the times listed above, larvae were anesthetized with buffered 0.001% MS222 (3-aminobenzoic acid ethyl ester methanesulfonate; Sigma, #E10521) and immediately viewed using fluorescence microscopy on an automated stage (Marianas imaging system, Intelligent