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Screening for chemicals that affect hair cell death and survival in the zebrafish lateral line

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Abstract

The zebrafish lateral line is an efficient model system for the evaluation of chemicals that protect and damage hair cells. Located on the surface of the body, lateral line hair cells are accessible for manipulation and visualization. The zebrafish lateral line system allows rapid screens of large chemical libraries, as well as subsequent thorough evaluation of interesting compounds. In this review, we focus on the results of our previous screens and the evolving methodology of our screens for chemicals that protect hair cells, and chemicals that damage hair cells using the zebrafish lateral line.

Keywords

zebrafish; lateral line; hair cells; chemical screen

Introduction

Hearing loss has been recognized by the World Health Organization (WHO) as a growing, serious global health concern (World Health Organization, 2010). In the United States alone, 15% of adults aged 20–69 report hearing impairment, and the prevalence of hearing impairment of seniors ranges from 60% to 90%, depending on the criteria used. In 2005, the WHO estimated that 278 million people worldwide had moderate to profound hearing loss, with 80% of them living in low and middle-income countries. While much work has been done to alleviate hearing loss through hearing aids or cochlear implants, prevention should be our ultimate goal. Developing methods to prevent hair cell death is an obvious mission and, when successful, will likely eliminate a large percent of hearing loss worldwide.

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Hearing loss prevention can come in a number of ways. Simply educating people worldwide about the dangers of noise exposure, chronic infection, and ototoxic injury would prevent a large percentage of hearing loss worldwide. In fact, the WHO estimates that roughly half of all cases of deafness and hearing loss would be avoidable through exposure prevention and early diagnosis. From a research standpoint, prevention can come by protecting hair cells and other cells of the inner ear from injury, and also by identifying agents that injure hair cells and supporting structures.

The zebrafish lateral line system offers a rapid and efficient model to evaluate the effects of large numbers of drugs on mechanosensory hair cells (Froehlicher et al., 2009; Coffin et al., 2010; Ou et al., 2010). Lateral line organs are composed of hair cells that share most properties with hair cells of the inner ear, including selective susceptibility to known ototoxins such as aminoglycoside antibiotics and chemotherapy agents (Williams and Holder, 2000; Ton et al., 2005; Harris et al., 2003; Ou et al., 2007). Their location on the body surface allows rapid analysis of the response to individual drugs and drug combinations. In this review, we will describe our experience using the zebrafish lateral line to screen and study chemicals that protect and damage hair cells of the inner ear.

Why screen for ototoxicity?

Ototoxins are drugs or other small molecules that cause inner ear damage. The number of well-established ototoxins is small for a number of reasons. For Food and Drug Administration (FDA) drug approval, most attention is focused appropriately on toxicity to larger organ systems such as the heart, liver, and kidney, but not the inner ear or hearing. In addition, there is no standard screen for ototoxicity. While drugs that cause dramatic hearing loss are ultimately identified (e.g. aminoglycosides and platinum drugs) due to severe patient symptoms, it is likely that there are a large number of drugs that cause milder degrees of hearing loss or slower onset of hearing and balance problems that go undetected, or are attributed to other causes. In young children, hearing loss after drug administration may be easily missed unless specifically screened for. In older adults, hearing loss after drug administration may more likely be attributed instead to age-related hearing loss. Furthermore, while we know of certain drugs that are ototoxic when administered alone, we know very little about the ototoxicity profiles of drug combinations and even less about the interactions of genetic variation and drug ototoxicities. Aminoglycosides cause synergistic ototoxicity with certain diuretics (Mathog et al., 1969; Brummett et al., 1981), but in reality this is a very rarely used combination. However, in this age of polypharmacy, it is likely that certain drug regimens are potentially dangerous to the inner ear. For these reasons, a forward, high throughput screen for ototoxic drugs would be of great value for the medical community and the pharmacological industry. With most in vivo model systems that are used to study hair cells, testing large numbers of drugs for toxicity is impractical, and the currently available cell lines do not adequately represent mature hair cells (Kalinec et al., 1999), which limits their applicability for revealing the fundamental pathways underlying ototoxicity in mature inner ears. We believe that chemical screening in the zebrafish lateral line offers a feasible way to test large numbers of drugs and drug combinations for toxicity to hair cells.

Why screen for protectants?

A large number of drugs and chemicals have been proposed to protect the inner ear against damage. These compounds include agents such as antioxidants, caspase inhibitors, and jun-kinase inhibitors (Matsui et al., 2002; Wang et al., 2003; Wang et al., 2004; Sha et al., 2006; Campbell et al., 2007; Eshraghi et al., 2007; Feldman et al., 2007). In particular, studies with aspirin as well as D-methionine have shown some promising evidence that they may be able

to partially prevent hearing loss in humans, and in some cases rescue hair cells from death (Sha et al., 2006; Campbell et al., 2007). However, very few drugs have made it to the point of clinical trials, none have consistently shown robust protection across a wide range of damage levels and none are yet approved by the FDA for use in the prevention of hearing loss or balance disorders.

Early cellular studies of hair cell death due to noise over-exposure and ototoxic drugs sought to characterize hair cell death as primarily apoptotic (Forge et al., 2000; Cunningham et al., 2002; Matsui et al., 2002; Cheng et al., 2003). It is now evident that both caspase-dependent and caspase-independent processes are involved in ototoxic drug-induced hair cell death (Jiang et al., 2006; Owens et al., 2009). Moreover, traditional concepts of cell death as apoptotic versus necrotic have been replaced by an understanding that apoptosis and necrosis are two extremes in a continuum of cell death mechanisms (reviewed in Vandenabeele et al., 2010), and that necrosis in some cases is a carefully regulated process similar to apoptosis (Holler et al., 2000; Degterev et al., 2005; Hitomi et al., 2008). Furthermore, there is significant interaction between death pathways (Zhang et al., 2009; reviewed in Zhivotovsky and Orrenius, 2010), such that inhibition of one cell death pathway can lead to activation of another. Specifically, inhibition of apoptotic pathways can lead to an increase in necrotic and autophagic death (Vercammen et al., 1998; Lin et al., 1999; Yu et al., 2004; Vandenabeele et al., 2006) and inhibition of autophagy can trigger apoptotic-like cell death (Boya et al., 2005). Put simply, a cell (in this case, a hair cell) can find a way to die even in the presence of a cell death inhibitor. These findings may partly explain why most protective drugs in the inner ear have demonstrated partial rather than complete protection. Therefore, in order to most completely and effectively block hair cell death, we are likely to need single drugs or combinations of drugs that block multiple aspects of cell death. Drug screening offers an efficient method by which large numbers of candidate protective drugs can be rapidly identified and tested alone or in combinations.

For practical reasons most studies of protection against inner ear damage use exposure to known ototoxic drugs or damaging noise levels. These are certainly the most straightforward damage paradigms that can be tested experimentally. One could imagine that a drug that prevents inner ear damage from noise or ototoxins might also be able to prevent progressive hearing loss in a young patient, or presbycusis in an older one. In fact, many necrotic and apoptotic pathways involved in ototoxin-induced hair cell death are also known to be involved in noise-induced hearing loss (Saunders et al., 1985; Nicotera et al., 2003; Yang et al., 2004; Han et al., 2006; reviewed in Op de Beeck et al., 2011) and age-related hearing loss (Usami et al., 1997; Sha et al., 2009; Someya et al., 2009).

Previous chemical screens in zebrafish

The zebrafish larva has numerous qualities that make it an ideal model organism for screening. First, the zebrafish's small size and high fecundity make it useful for examining large numbers of treatment conditions. Second, the optically clear body facilitates phenotypic evaluation of a number of organ systems, including, but not limited to the heart, nervous system, eyes, ears, and lateral line hair cells. The zebrafish genome is also very well characterized and manipulated (Ekker et al., 2007), for example, using morpholino oligonucleotides to knockdown molecular targets (Nasevicius and Ekker, 2000) and through the generation of transgenic zebrafish (Stuart et al., 1988; Stuart et al., 1990). The use of the zebrafish in a chemical screen was first demonstrated by Peterson et al., (2000), who identified small molecules that modulate development of the cardiovascular system, central nervous system, neural crest, and ear. Subsequent chemical screens have identified a number of different compounds, including novel retinoids (Sachidanandan et al., 2008), drugs that affect heart rate (Milan et al., 2003), drugs that regulate haematopoietic stem cell

homeostasis (North et al., 2007), compounds that inhibit melanoma (White et al., 2011) and chemicals that can suppress a genetic mutation that causes coarctation of the aorta in a zebrafish model (Peterson et al., 2004).

Hair cells of the lateral line can be rapidly assessed

In all aquatic vertebrates, the hair cells of the lateral line are external and directly exposed to the water surrounding the fish, where they are used to detect direction and changes in flow (Figure 1). At five days post-fertilization (dpf), the larval zebrafish is approximately 3 mm in length. While a number of investigators had previously demonstrated that lateral line hair cells are susceptible to aminoglycoside exposure (Kaus, 1987; Song et al., 1995), in vivo imaging of individual zebrafish lateral line hair cells was first demonstrated by Williams and Holder (2000), who observed neomycin-induced hair cell death and regeneration in larval zebrafish. Harris et al. (2003) subsequently developed additional assays to quantify hair cell death and regeneration in the lateral line, laying the groundwork for subsequent genetic and chemical screening studies aimed at discovering modulators of hair cell sensitivity to ototoxic exposures and for further toxicity screening. Additional work by our group and others (Murakami et al., 2003; Ton et al., 2005; Santos et al., 2006; Ou et al., 2007; Owens, 2007; Coffin et al., 2009) has characterized the response of the zebrafish lateral line to ototoxic compounds such as aminoglycosides and cisplatin.

One great strength of the zebrafish lateral line model has been the ability to vary damage protocol parameters in large-scale experiments. An entire two-dimensional dose response matrix (with variable doses of protectant versus variable doses of ototoxic drug) is easily obtained due to the availability of large numbers of zebrafish and the efficiency of testing. This kind of testing can reveal important differences that are not otherwise readily identified in other systems (e.g. protection against only a limited range of ototoxin, or toxicity of the protectant at higher doses). A second strength for our purposes is that hair cell death occurs fairly rapidly, with near total hair cell death possible after 1 hour of neomycin and 6 hours of cisplatin (Harris et al., 2003; Ou et al., 2007). The speed of these events is critical to the efficiency of zebrafish screens. Lastly, the lateral line hair cells are studied within a whole organism, and as a result are in their true native environment, including proper cell polarity, afferent and efferent nerve connections, and intimate association with neighboring supporting cells.

Fluorescent dyes

Critical to in vivo studies of the zebrafish lateral line is the ability of hair cells to selectively take up a number of fluorescent dyes (Santos et al., 2006). Some of these deserve special mention for particular utility in screening. YO-PRO1 (Invitrogen) is a cyanine dye which binds to DNA and very brightly labels hair cell nuclei (Figure 2). The staining is easily visualized for over 24 hours and thus allows long-term imaging, or longer duration screens.

FM1-43 (Invitrogen) is well known as a cytoplasmic/plasma membrane label of hair cells in a variety of animal models. Staining appears to be mechanotransduction dependent (Gale et al., 2001). It labels lateral line hair cells brightly. In some cases, however, FM1-43 staining can be problematic for screening, due to time-dependent variation in its staining pattern. Initially, staining is primarily around the apices of the hair cells. Over time, the staining becomes more membrane-bound and vesicular. This variation in labeling can convolute attempts to rapidly evaluate hair cells during a screen.

DASPEI has also been extensively used in our lab and can be used to rapidly label hair cells and quantify damage. Its labeling is dependent on an intact mitochondrial potential. DASPEI

is particularly instrumental to our genetic screens, where evaluation of large numbers of larval zebrafish in a single day is necessary (Owens et al., 2008).

While a number of other dyes, such as Lysotracker (Invitrogen), Mitotracker (Invitrogen), and DASPEI are available and have been used successfully (Owens et al., 2007; Owens et al., 2008;) in zebrafish, for most in vivo hair cell survival screens in our laboratory, a simple nuclear stain such as YO-PRO1 is sufficient. The nuclear stain allows assessment of subtle changes in nuclear morphology as well as more dramatic changes such as nuclear condensation and fragmentation. However, for dose response testing, immunohistochemistry allows for quantitative hair cell counts that can be difficult to perform in vivo. Typically, we have used anti-parvalbumin antibody to label lateral line hair cells in fixed tissue (Figure 2). Lastly, for analysis of aminoglycoside uptake, Texas Red-conjugated gentamicin (GTTR; (Steyger et al., 2003)) is very rapidly taken up by hair cells of the lateral line and is easily visualized with in vivo fluorescence microscopy (Figure 2).

Disadvantages of the zebrafish

It is important to note some critical differences between zebrafish lateral line hair cells and mammalian inner ear hair cells. Hair cells of the lateral line essentially extend their stereocilia and kinocilia into the surrounding media. There is no separation of fluid spaces analogous to those of the inner ear (Figure 1). Inner ear hair cells also come in several varieties (e.g. inner and outer hair cells) with distinct susceptibilities to ototoxins. Overall, any discovery made in fish must be confirmed in mammals prior to translational applications. However, the zebrafish lateral line model permits rapid identification of chemicals of interest, as well as large-scale follow-up studies to carefully examine subtleties in how a chemical damages or protects hair cells.

CHEMICAL SCREENING FOR HAIR CELL PROTECTION

Our chemical screens have initially focused on protection, particularly against aminoglycoside-induced hair cell death. While different ototoxins likely activate different death pathways, it is also likely that there is overlap and convergence. Our hope is that a chemical or combination of chemicals found to be protective against aminoglycoside-induced hair cell death will also have potential applications against other causes of hair cell injury.

ChemBridge DIVERSet E small molecule library protection screen

Our initial chemical screen assessed a subset of the ChemBridge DIVERSet E library for small molecules that inhibit neomycin-induced hair cell death (Owens et al., 2008). This library consists of structurally diverse molecules without previous known bioactivity that are designed to conform to the Lipinski “Rules of 5” to optimize their “drug-like” potential (Lipinski et al., 2001). The experimental protocol involved labeling 5 dpf zebrafish with YO-PRO1 and FM1-43, then placing one fish into each well of a 96-well plate with an optical grade base for microscopy (Figure 3). Each well was then pretreated with drugs from the small molecule library for 1 hour. After pretreatment, neomycin was added to each well at a concentration that causes death of almost all hair cells in each lateral line neuromast. After neomycin treatment, the 96-well plate was then placed directly onto a motorized stage of an inverted epifluorescent microscope. Hair cell survival could be rapidly assessed. For any wells that demonstrated robust protection, the results were retested and confirmed.

We screened 10,960 compounds from this library, yielding two “hits” with robust protection (Owens et al., 2008). Interestingly, both hits (hereafter named PROTO1 and PROTO2) share a similar chemical structure and are of a family of compounds called ureathiophene

carboxamides. Follow-up dose response testing of both compounds showed that both compounds demonstrated dose-dependent protection against neomycin damage. More importantly, both compounds protected against a broad range of neomycin doses – in other words, the protective effects did not disappear at high doses of neomycin. Microbiological testing revealed that neither compound interferes with the bactericidal and bacteriostatic effects of neomycin. Subsequent testing in a mouse utricle in vitro preparation demonstrated similar levels of protection, showing for the first time that a hair cell protective compound found in a zebrafish could have results which translate into mammalian findings. PROTO1 is currently in early stages of drug development. Recent studies have demonstrated that PROTO1 can protect against hearing loss induced by kanamycin-induced hair cell toxicity in vivo in the rat (Rubel et al., 2011).

NINDS Custom Collection II library protection screen

The majority of uncharacterized small molecules are not likely to have any biological activity. For those that do cause a phenotypic change, the molecular targets are obviously unknown. Thus, small molecules are not yet “drugs”, and the number of steps required for one to be developed into an FDA-approved drug is numerous. A second approach we have undertaken is screening libraries of FDA-approved drugs and bioactives (Ou et al., 2009). The NINDS Custom Collection II (Microsource) is a library of 1,040 FDA approved drugs and other compounds with known biological activity. The advantage is that any drug identified as promising, already has known targets and bioactivity, and typically has been used safely in humans.

We screened this library using a similar screening protocol and identified seven drugs that demonstrated robust protection on initial screening. These seven compounds were amsacrine, carvedilol, cepharanthine, drofenine, hexamethylenamiloride, phenoxybenzamine, and tacrine. As with PROTO1 and 2, all seven compounds demonstrated dose-dependent protection against neomycin, as well as protection against a wide range of neomycin concentrations. None interfered with the bactericidal and bacteriostatic effects of neomycin. We also tested whether each of these drugs affected aminoglycoside uptake using Texas Red-conjugated gentamicin (GTTR). Interestingly, four of the seven drugs (amsacrine, carvedilol, hexamethylenamiloride, and phenoxybenzamine) appeared to reduce uptake of GTTR. The remaining three drugs did not, and thus likely have intracellular targets responsible for their protective activity. It should be noted that blocking uptake of aminoglycosides or inhibiting intracellular targets would both be of potential clinical utility as long as the effects are limited to hair cells. In fact, given the number of possible death pathways triggered by ototoxic agents, blocking uptake might be the most definitive way to prevent hair cell death.

In summary, we have been able to use this approach to identify FDA-approved drugs that reliably block neomycin-induced hair cell death, facilitating the transition to clinical translation. The anticholinergic drug tacrine, used for the treatment of Alzheimer’s dementia, has since been found to have similar protective effects in vitro on hair cells of the mouse utricle (Ou et al., 2009).

The ChemBridge DIVERSet E library of small molecules and the NINDS Custom Collection II represent the first libraries screened by our group for hair cell protection against neomycin. Many other small molecule and bioactives libraries are available, and screens of these libraries for aminoglycoside and cisplatin protection are ongoing.

Screening the zebrafish lateral line for protection against aminoglycoside-induced hair cell death has quickly yielded a large number of compounds that warrant further evaluation. We would expect a subset of these compounds to have similar effects in mammals, and a subset

to have effects that may extend to other causes of ototoxic injury (e.g. platinum drugs, noise, and perhaps aging).

CHEMICAL SCREENING FOR HAIR CELL TOXICITY

Screening for chemicals that protect hair cells has provided a number of interesting protective compounds. A simple modification of the protection screen transforms it into a screen for chemicals that are damaging to hair cells of the zebrafish lateral line. While any potential hair cell toxin must then be confirmed in mammalian systems, identifying chemicals that cause selective hair cell toxicity in a living organism is a promising method for identifying potential ototoxins.

NINDS Custom Collection II library toxicity screen

The value of screening a small molecule library for hair cell toxicity is limited. We thus chose to perform our first hair cell toxicity screen on the NINDS Custom Collection II library of 1,040 FDA-approved compounds and bioactives (Chiu et al., 2008). By simply removing the aminoglycoside exposure from the protection screen protocol, we could easily monitor for hair cell toxicity (Figure 3). Because neomycin causes hair cell death in the 100 μ M concentration range, we chose to screen for hair cell toxicity using a similar concentration. In brief, hair cells of five dpf zebrafish were first labeled with YO-PRO1 to label hair cell nuclei. One zebrafish was then placed into each well of a 96-well plate. The zebrafish were then treated for one hour with drugs from the NINDS Custom Collection II at 100 μ M concentration for one hour. The 96-well plate was then imaged directly with fluorescence microscopy, as described for the hair cell protection screens.

This screen identified 21 confirmed hits. As proof of concept, seven of the 21 hits were known ototoxins, including neomycin and cisplatin. The remaining 14 drugs were not known to have ototoxic effects. To examine whether the findings were translatable to mammalian inner ears, we tested two of the drugs, propantheline and pentamidine, in vitro on free floating cultures of the mouse utricle. This testing revealed similar hair cell toxicity in the mouse utricle, with significant hair cell loss at doses lower than those required with aminoglycosides in identical experimental conditions. However, neither the zebrafish lateral line nor the mammalian utricle in vitro reveal a drug's inner ear penetration when given systemically – a matter that is critical to a drug's ototoxic potential. To truly demonstrate ototoxicity, testing must be performed in vivo in mammals. These studies are ongoing.

National Cancer Institute (NCI) Approved Oncology Drugset

The screen in the NINDS Custom Collection II was performed to demonstrate feasibility of the hair cell toxicity screen in the zebrafish lateral line. It was not meant to identify all drugs in the library that cause hair cell death. Drugs were only screened at one concentration (100 μ M) for a treatment period of single duration (one hour). While seven known ototoxins were identified, five were not. This is likely because exposure parameters were not sufficient to result in hair cell damage. We know from other studies (Ou et al., 2007; Owens et al., 2009) that hair cell death from cisplatin and other aminoglycosides can require a minimum of 6 hours of exposure. In addition, drugs that were lethal to the zebrafish were not retested. It is certainly possible that some of these drugs, when tested at lower doses would demonstrate hair cell death without being lethal to the fish.

Our subsequent screen for hair cell toxicity had different goals. We wished to perform a more comprehensive screen – i.e. determine whether we could detect all known (as well as novel) ototoxins in a drug library. We elected to screen the NCI Approved Oncology Drugset, a library composed of 88 FDA-approved anti-cancer drugs (Hirose et al., 2011). Anti-cancer drugs are particularly fertile ground for ototoxicity research for multiple

reasons. By nature, many are cytotoxic and have good tissue penetration and thus would have a higher risk of ototoxicity. Secondly, they are typically used in combination and are therefore useful for the study of synergistic ototoxicity. Lastly, patients on anti-cancer drugs are often well-monitored and thus hearing loss is perhaps more likely to be detected. This last point comes with the caveat that many cancer studies report only severe hearing loss as a complication, leaving out mild and moderate hearing loss that most patients would consider significant.

To make the screen more comprehensive, we addressed some of the deficiencies of the first hair cell toxicity screen – namely, that only one exposure paradigm was used, and that lethal drugs were not retested. For the anti-cancer drug screen, we tested all drugs at a 100 μ M concentration with exposure times of both 1 hour and 6 hours. If an exposure was found to be lethal to the fish, we reduced the dose by half and retested at both durations until we had reached a dose that was nonlethal and the hair cells could be assessed.

Using this modified protocol, the screen successfully identified 4 out of 5 (80%) of known ototoxins (Hirose et al., 2011). Interestingly, the known ototoxin carboplatin was not identified. To determine whether this was in fact a “true miss” of the screening protocol, or whether other factors were contributing, we retested carboplatin and were unable to cause hair cell damage even with 1 mM carboplatin for 24 hours. This may represent a difference in the carboplatin pharmacokinetics between zebrafish and mammals. It is interesting to note, however, that in a study by Dean et al. (Dean et al., 2008), carboplatin-treated patients had only a 4% incidence of hearing loss compared to 57% of cisplatin-treated patients. It is possible that this difference may be seen in the zebrafish lateral line as well.

The anti-cancer drug family is also interesting because while very few are well-established ototoxins, many could be termed “suspected” ototoxins. These drugs have had isolated case reports of hearing loss after drug exposure (Tibaldi et al., 1998; Moss et al., 1999; Attili et al., 2008). In many of these cases, the drugs were given in combination with other drugs, so it is unclear if an individual drug or a combination was responsible for the hearing loss. The anti-cancer drug screen detected 4 out of 7 (57%) “suspected” ototoxins suggesting that some of these drugs might in fact be true ototoxins and merit further evaluation. These four drugs were subsequently found to cause dose-dependent hair cell loss in lateral line hair cells. In vivo testing in mammals is currently in progress.

Synergistic hair cell toxicity

As stated previously, the efficiency of testing in the zebrafish permits easy manipulation of exposure parameters and evaluation of damage. This is particularly valuable in the investigation of synergistic ototoxicity. The concept of synergistic ototoxicity is not new. Aminoglycosides are known to have synergistic ototoxicity with loop diuretics, as well as noise (Mathog et al., 1969; Brummett et al., 1981; Santi et al., 1982; Li et al., 2009). These interactions however are quite rare in clinical practice. In contrast, it is the norm for anti-cancer drugs to be used in combination. Therefore, we sought to examine a number of anti-cancer drug combinations for synergistic hair cell toxicity (Hirose et al., 2011).

We utilized low, relatively non-toxic concentrations of each anti-cancer drug (i.e. concentrations for each drug that individually cause minimal hair cell damage) in order to facilitate the identification of synergistic effects. Multiple commonly used anti-cancer drug combinations with cisplatin were evaluated. We chose to evaluate for synergistic effects by calculating Chou-Talalay combination indices for each group of drugs tested (Chou et al., 1984). This combination index is a commonly used method for analyzing drug interactions, and is widely used in the cancer literature. Of the ten combinations evaluated, five of them demonstrated significant synergism. In particular, the combinations of cisplatin with

vinorelbine, cisplatin with vincristine, and cisplatin with doxorubicin were considered strongly synergistic (Hirose et al., 2011).

With the exception of this report, we have screened only individual drugs for hair cell toxicity. Our findings of synergistic hair cell toxicity using cancer therapeutics suggest that in some scenarios, drug combinations may be even more important than toxicity from single drugs. While only anti-cancer drugs were tested in this study, this finding likely applies by some extent to all drugs. This suggests that when considering a patient's risk for an ototoxic injury, their entire drug regimen, rather than individual drugs should be considered. While drug screens of all possible combinations of drugs is impractical, screens of particularly common drug regimens might be warranted.

In summary, we have used the zebrafish lateral line to screen multiple drug libraries for hair cell toxicity. We have also found that certain drug combinations may have more ototoxic potential than others. As stated previously, to truly prove ototoxicity, in vivo testing in mammals is paramount. However, our hope is that the zebrafish can be used as an early screen for ototoxicity to identify potentially dangerous drugs or drug combinations that merit more formal audiologic testing during clinical trials.

TECHNICAL CONSIDERATIONS FOR CHEMICAL SCREENS IN THE LATERAL LINE

Selecting a damage protocol

There are a number of technical considerations to be aware of when using zebrafish lateral line hair cells for chemical screens. Parameters for screening will obviously depend on the purpose of the screen. To identify potential therapeutic drugs aimed at protecting hair cells, a robust and invariant damaging paradigm is needed. Typically the lowest dose of damaging agent that still gives maximum damage without systemic lethality is used. Short incubation times are important to speed sample throughput, however, to identify possible new ototoxic drugs, longer incubation times may be needed to identify damage. Relative damage caused by chemicals within a library can be influenced by the carrier in which chemicals are dissolved. Libraries are typically dissolved in dimethylsulfoxide (DMSO), which has been shown to be toxic to hair cells in some models (Qi et al., 2008) and can be lethal to zebrafish at too high a concentration. As a result, carrier-only controls are needed during each screening experiment to interpret results.

Library selection

The choice of the type of chemical library will influence screen design. Diverse small molecule libraries have the advantage that any discovery has the potential to be developed into a novel drug. Not surprisingly, the "hit rate" for small molecule screens is relatively low because the vast majority of small molecules have no obvious measurable bioactivity. In contrast, screens of previously developed drugs or bioactives typically have higher hit rates (Rothstein et al., 2005; Ouertatani-Sakouhi et al., 2010; Cho et al., 2011). In our hands, screening a library of FDA-approved drugs and bioactives for protection against neomycin damage had a 35-fold higher success rate than screening a diverse small molecule library of unknown activity (Owens et al., 2008; Ou et al., 2009). For screens of larger libraries, multiplexing (screening multiple drugs/chemicals per well) is a common practice to reduce the overall number of plates needed to be screened. Since many of the compounds in small molecule libraries have little activity, the chances of confounding interactions are miniscule, although certainly interactions between small molecules, or lethality of certain molecules can potentially mask a positive effect. Confounding interactions will be more likely while

screening libraries of known drugs and bioactives; however the relatively small size of these libraries usually makes multiplexing unnecessary.

Another consideration is the criteria for selection of initial hits for subsequent evaluation. When screening for compounds that protect hair cells against damage, we have used criteria that are relatively stringent, particularly for large libraries. By contrast, our criteria are considerably relaxed in screens designed to identify potentially toxic compounds. False negative identifications are less desirable, and a higher false positive rate is tolerable because these can be easily retested. Confirmatory testing is critical for any “hit”. Typically, this consists of repeat testing in triplicate, followed by full dose-response testing. Typically, a high false positive rate would make confirmatory testing frequent and onerous, however, the efficiency of the zebrafish model allows this kind of testing to be done rapidly. For protectants, formal two-dimensional dose-response matrices are obtained for each protectant. For example, with neomycin we first determine the optimal protective concentration against the minimum concentration of neomycin that causes maximal hair cell loss. We then test this concentration of the potential protectant against a wide range of neomycin concentrations, from a concentration that has no activity to a level at least 4X our original concentration. For toxicants, a simple dose response curve with increasing doses of the potential toxic drug is performed.

Following up hits from screens

An important consideration is that our screening experiments represent a phenotypic approach. Determining mechanisms of action for identified chemicals is challenging. Even drugs with known bioactivity and known targets are expected to have several unidentified targets. One way to test this idea is to examine other drugs with similar known activities.

A common mechanism of action for protection against aminoglycoside damage is reduction of aminoglycoside uptake (Ou et al., 2009). Therefore, as part of our standard evaluation of candidate protectants, we quantitatively measure uptake of fluorescently conjugated gentamicin in the presence or absence of the candidate protective drug (Figure 2).

Finally, one unwanted mechanism of action of new compounds that protect against ototoxicity of therapeutic drugs is interference with their desired therapeutic targets. Thus, all protectants against aminoglycosides are tested to determine whether they impair the bactericidal and bacteriostatic activities of aminoglycosides. Similarly, all protectants against cisplatin are tested to determine whether they impair the tumoricidal activity of cisplatin against cancer cell lines.

Mammalian testing

A critical step in evaluating compounds revealed by zebrafish screens is confirmation of activity in mammals. One assay to confirm findings is to test compounds in vitro in the mature mouse utricle, used instead of cochlea because of inherent difficulties culturing that tissue. Using this preparation, the lateral line hair cell protection seen with the small molecule PROTO-1, and the FDA-approved drug tacrine, has been confirmed in the mammalian utricle in vitro (Owens et al., 2008; Ou et al., 2009). We also have tested candidate ototoxins identified using the zebrafish in the mouse utricle and found similar findings (Chiu et al., 2008) in vitro. However, survival of mature inner ear hair cell preparations in vitro can be tenuous and affected by other factors such as culture media pH and ionic concentrations that may be affected by candidate protective or toxic drugs. More recently, we have moved more rapidly towards in vivo testing in rats (Rubel et al., 2011). This preparation more closely represents the clinical situation. Delivering a drug to the water a fish is swimming in, or to the culture media hair cells are bathed in does not closely model

the separation of fluid spaces characteristic of the inner ear, or simulate any issues of drug penetration to the inner ear.

Conclusion

The zebrafish lateral line is a unique and promising system that allows rapid and efficient in vivo evaluation of hair cells. As demonstrated in this review, chemical screening for drugs that affect hair cell survival is an evolving and dynamic process. With each new screen come changes and modifications in screening parameters and methodology. Screening for protection against a variety of ototoxic insults is possible, as is screening for other causes of hair cell toxicity. Subsequent testing in the zebrafish can then be used to elucidate interesting biological findings about hair cell survival and death with potential translational impact.

References

- Attili VS, Bapsy PP, Anupama G, Lokanatha D. Irreversible sensorineural hearing loss due to Imatinib. *Leuk Res.* 2008; 32:991–992. [PubMed: 18221994]
- Boya P, Gonzalez-Polo RA, Casares N, Perfettini JL, Dessen P, Larochette N, Metivier D, Meley D, Souquere S, Yoshimori T, Pierron G, Codogno P, Kroemer G. Inhibition of macroautophagy triggers apoptosis. *Mol Cell Biol.* 2005; 25:1025–40. [PubMed: 15657430]
- Brummett RE, Bendrick T, Himes D. Comparative ototoxicity of bumetanide and furosemide when used in combination with kanamycin. *J Clin Pharmacol.* 1981; 21:628–36. [PubMed: 7338574]
- Campbell KC, Meech RP, Klemens JJ, Gerber MT, Dyrstad SS, Larsen DL, Mitchell DL, El-Azizi M, Verhulst SJ, Hughes LF. Prevention of noise- and drug-induced hearing loss with D-methionine. *Hear Res.* 2007; 226:92–103. [PubMed: 17224251]
- Cheng AG, Cunningham LL, Rubel EW. Hair cell death in the avian basilar papilla: characterization of the in vitro model and caspase activation. *J Assoc Res Otolaryngol.* 2003; 4:91–105. [PubMed: 12417974]
- Chiu LL, Cunningham LL, Raible DW, Rubel EW, Ou HC. Using the zebrafish lateral line to screen for ototoxicity. *J Assoc Res Otolaryngol.* 2008; 9:178–90. [PubMed: 18408970]
- Cho Y, Vermeire JJ, Merkel JS, Leng L, Du X, Bucala R, Cappello M, Lolis E. Drug repositioning and pharmacophore identification in the discovery of hookworm MIF inhibitors. *Chem Biol.* 2011; 18(9):1089–101. [PubMed: 21944748]
- Chou TC, Talalay P. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. *Adv Enzyme Regul.* 1984; 22:27–55. [PubMed: 6382953]
- Coffin AB, Reinhart KE, Owens KN, Raible DW, Rubel EW. Extracellular divalent cations modulate aminoglycosides-induced hair cell death in the zebrafish lateral line. *Hear Res.* 2009; 253:42–51. [PubMed: 19285547]
- Coffin AB, Ou H, Owens KN, Santos F, Simon JA, Rubel EW, Raible DW. Chemical screening for hair cell loss and protection in the zebrafish lateral line. *Zebrafish.* 2010; 7:3–11. [PubMed: 20192852]
- Cunningham LL, Cheng AG, Rubel EW. Caspase activation in hair cells of the mouse utricle exposed to neomycin. *J Neurosci.* 2002; 22:8532–40. [PubMed: 12351727]
- Dean JB, Hayashi SS, Albert CM, King AA, Karzon R, Hayashi RJ. Hearing loss in pediatric oncology patients receiving carboplatin-containing regimens. *J Pediatr Hematol Oncol.* 2008; 30:130–4. [PubMed: 18376265]
- Degterev A, Huang Z, Boyce M, Li Y, Jagtap P, Mizushima N, Cuny GD, Mitchison TJ, Moskowitz MA, Yuan J. Chemical inhibitor of nonapoptotic cell death with therapeutic potential for ischemic brain injury. *Nat Chem Biol.* 2005; 1(2):112–9. [PubMed: 16408008]
- Ekker SC, Stemple DL, Clark M, Chien CB, Rasooly RS, Javois LC. Zebrafish genome project: bringing new biology to the vertebrate genome field. *Zebrafish.* 2007; 4:239–51. [PubMed: 18284331]

- Eshraghi AA, Adil E, He J, Graves R, Balkany TJ, Van De Water TR. Local Dexamethasone Therapy Conserves Hearing in an Animal Model of Electrode Insertion Trauma-Induced Hearing Loss. *Otol Neurotol*. 2007
- Feldman L, Efrati S, Eviatar E, Abramssohn R, Yarovoy I, Gersch E, Averbukh Z, Weissgarten J. Gentamicin-induced ototoxicity in hemodialysis patients is ameliorated by N-acetylcysteine. *Kidney Int*. 2007; 72:359–63. [PubMed: 17457375]
- Forge A, Li L. Apoptotic death of hair cells in mammalian vestibular sensory epithelia. *Hear Res*. 2000; 139:97–115. [PubMed: 10601716]
- Froehlicher M, Liedtke A, Groh KJ, Neuhauss SC, Segner H, Eggen RI. Zebrafish (*Danio rerio*) neuromast: promising biological endpoint linking developmental and toxicological studies. *Aquat Toxicol*. 2009; 95:307–19. [PubMed: 19467721]
- Gale JE, Marcotti W, Kennedy HJ, Kros CJ, Richardson GP. FM1-43 dye behaves as a permeant blocker of the hair-cell mechanotransducer channel. *J Neurosci*. 2001; 21:7013–25. [PubMed: 11549711]
- Han W, Shi X, Nuttall AL. AIF and endoG translocation in noise exposure induced hair cell death. *Hear Res*. 2006; 211:85–95. [PubMed: 16309861]
- 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. [PubMed: 12943374]
- Hirose Y, Simon JA, Ou HC. Hair Cell Toxicity in Anti-cancer Drugs: Evaluating an Anti-cancer Drug Library for Independent and Synergistic Toxic Effects on Hair Cells Using the Zebrafish Lateral Line. *J Assoc Res Otolaryngol*. 2011
- Hitomi J, Christofferson DE, Ng A, Yao J, Degterev A, Xavier RJ, Yuan J. Identification of a molecular signaling network that regulates a cellular necrotic cell death pathway. *Cell*. 2008; 135(7):1311–23. [PubMed: 19109899]
- Holler N, Zaru R, Micheau O, Thome M, Attinger A, Valitutti S, Bodmer JL, Schneider P, Seed B, Tschoep J. Fas triggers an alternative, caspase-8-independent cell death pathway using the kinase RIP as effector molecule. *Nat Immunol*. 2000; 1(6):489–95. [PubMed: 11101870]
- Jiang H, Sha SH, Forge A, Schacht J. Caspase-independent pathways of hair cell death induced by kanamycin in vivo. *Cell Death Differ*. 2006; 13:20–30. [PubMed: 16021180]
- Kalinec F, Kalinec G, Boukhvalova M, Kachar B. Establishment and characterization of conditionally immortalized organ of Corti cell lines. *Cell Biol Int*. 1999; 23:175–84. [PubMed: 10562438]
- Kaus S. The effect of aminoglycoside antibiotics on the lateral line organ of *Aplocheilus lineatus* (Cyprinodontidae). *Acta Otolaryngol*. 1987; 103:291–8.
- Li H, Steyger PS. Synergistic ototoxicity due to noise exposure and aminoglycoside antibiotics. *Noise Health*. 2009; 11:26–32. [PubMed: 19265251]
- Lin Y, Devin A, Rodriguez Y, Liu ZG. Cleavage of the death domain kinase RIP by caspase-8 prompts TNF-induced apoptosis. *Genes Dev*. 1999; 13:2514–26. [PubMed: 10521396]
- 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. [PubMed: 11259830]
- Mathog RH, Klein WJ Jr. Ototoxicity of ethacrynic acid and aminoglycoside antibiotics in uremia. *N Engl J Med*. 1969; 280:1223–4. [PubMed: 5767464]
- Matsui JJ, Ogilvie JM, Warchol ME. Inhibition of caspases prevents ototoxic and ongoing hair cell death. *J Neurosci*. 2002; 22:1218–27. [PubMed: 11850449]
- Milan DJ, Peterson TA, Ruskin JN, Peterson RT, MacRae CA. Drugs that induce repolarization abnormalities cause bradycardia in zebrafish. *Circulation*. 2003; 107:1355–8. [PubMed: 12642353]
- Moss PE, Hickman S, Harrison BR. Ototoxicity associated with vinblastine. *Ann Pharmacother*. 1999; 33:423–425. [PubMed: 10332533]
- Murakami SL, Cunningham LL, Werner LA, Bauer E, Pujol R, Raible DW, Rubel EW. Developmental differences in susceptibility to neomycin-induced hair cell death in the lateral line neuromasts of zebrafish (*Danio rerio*). *Hear Res*. 2003; 186:47–56. [PubMed: 14644458]

- Nasevicius A, Ekker SC. Effective targeted gene “knockdown” in zebrafish. *Nat Genet.* 2000; 26:216–220. [PubMed: 11017081]
- Nicotera TM, Hu BH, Henderson H. The caspase pathway in noise-induced apoptosis of the chinchilla cochlea. *J Assoc Res Otolaryngol.* 2003; 4:466–477. [PubMed: 14534835]
- North TE, Goessling W, Walkley CR, Lengerke C, Kopani KR, Lord AM, Weber GJ, Bowman TV, Jang IH, Grosser T, Fitzgerald GA, Daley GQ, Orkin SH, Zon LI. Prostaglandin E2 regulates vertebrate haematopoietic stem cell homeostasis. *Nature.* 2007; 447:1007–11. [PubMed: 17581586]
- Op de Beeck K, Schacht J, Van Camp G. Apoptosis in acquired and genetic hearing impairment: the programmed death of the hair cell. *Hear Res.* 2011; 281:18–27. [PubMed: 21782914]
- Ou HC, Raible DW, Rubel EW. Cisplatin-induced hair cell loss in zebrafish (*Danio rerio*) lateral line. *Hear Res.* 2007; 233:46–53. [PubMed: 17709218]
- Ou HC, Santos F, Raible DW, Simon JA, Rubel EW. Drug screening for hearing loss: using the zebrafish lateral line to screen for drugs that prevent and cause hearing loss. *Drug Discov Today.* 2010; 15:265–71. [PubMed: 20096805]
- Ou HC, Cunningham LL, Francis SP, Brandon CS, Simon JA, Raible DW, Rubel EW. 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. [PubMed: 19241104]
- Quertatani-Sakouhi H, El-Turk F, Fauvet B, Cho MK, Pinar Karpinar D, Le Roy D, Dewor M, Roger T, Bernhagen J, Calandra T, Zweckstetter M, Lashuel HA. Identification and characterization of novel classes of macrophage migration inhibitory factor (MIF) inhibitors with distinct mechanisms of action. *J Biol Chem.* 2010; 285(34):26581–98. [PubMed: 20516071]
- Owens KN, Coffin AB, Hong LS, Bennett KO, Rubel EW, Raible DW. Response of mechanosensory hair cells of the zebrafish lateral line to aminoglycosides reveals distinct cell death pathways. *Hear Res.* 2009; 253:32–41. [PubMed: 19285126]
- Owens KN, Santos F, Roberts B, Linbo T, Coffin AB, Knisely AJ, Simon JA, Rubel EW, Raible DW. Identification of genetic and chemical modulators of zebrafish mechanosensory hair cell death. *PLoS Genet.* 2008; 4:e1000020. [PubMed: 18454195]
- Owens KNCDEMGREWRDWPR. Ultrastructural analysis of aminoglycoside-induced hair cell death in the zebrafish lateral line reveals an early mitochondrial response. *Journal of Comparative Neurology.* 2007 In press.
- Peterson RT, Link BA, Dowling JE, Schreiber SL. Small molecule developmental screens reveal the logic and timing of vertebrate development. *Proc Natl Acad Sci U S A.* 2000; 97:12965–9. [PubMed: 11087852]
- Peterson RT, Shaw SY, Peterson TA, Milan DJ, Zhong TP, Schreiber SL, MacRae CA, Fishman MC. Chemical suppression of a genetic mutation in a zebrafish model of aortic coarctation. *Nat Biotechnol.* 2004; 22:595–9. [PubMed: 15097998]
- Qi W, Ding D, Salvi RJ. Cytotoxic effects of dimethyl sulfoxide (DMSO) on cochlear organotypic cultures. *Hear Res.* 2008; 236:52–60. [PubMed: 18207679]
- Rothstein JD, Patel S, Regan MR, Haenggeli C, Huang YH, Bergles DE, Jin L, Dykes Hoberg M, Vidensky S, Chung DS, Toan SV, Bruijn L, Su ZZ, Gupta P, Fisher PB. Beta-lactam antibiotics offer neuroprotection by increasing glutamate transporter expression. *Nature.* 2005; 433(7021):73–7. [PubMed: 15635412]
- Rubel, E.; Robbins, C.; Owens, K.; Raible, D.; Simon, J. PROTO1 provides robust protection against kanamycin-induced hearing loss in rats. Midwinter Research Meeting of the Association for Research in Otolaryngology; Baltimore, MD. 2011.
- Sachidanandan C, Yeh JR, Peterson QP, Peterson RT. Identification of a novel retinoid by small molecule screening with zebrafish embryos. *PLoS One.* 2008; 3:e1947. [PubMed: 18398471]
- Santi PA, Ruggero MA, Nelson DA, Turner CW. Kanamycin and bumetanide ototoxicity: anatomical, physiological and behavioral correlates. *Hear Res.* 1982; 7:261–79. [PubMed: 7118731]
- Santos, F.; McDonald, G.; Rubel, EW.; Raible, DW. Lateral line hair cell maturation is a determinant of aminoglycoside susceptibility in zebrafish (*Danio rerio*). *Hearing Research*; 2006.

- Saunders JC, Dear SP, Schneider ME. The anatomical consequences of acoustic injury: a review and tutorial. *J Acoust Soc Am*. 1985; 78:833–860. [PubMed: 4040933]
- Sha SH, Qiu JH, Schacht J. Aspirin to prevent gentamicin-induced hearing loss. *N Engl J Med*. 2006; 354:1856–7. [PubMed: 16641409]
- Sha SH, Chen FQ, Schacht J. Activation of cell death pathways in the inner ear of the aging CBA/J mouse. *Hear Res*. 2009; 254:92–99. [PubMed: 19422898]
- Someya S, Xu J, Kondo K, Ding D, Salvi RJ, Yamasomba T, Rabinovitch PS, Weindrich R, Leeuwenburgh C, Tanokura M, Prolla TA. Age-related hearing loss in C57BL/6J mice is mediated by Bak-dependent mitochondrial apoptosis. *Proc Natl Acad Sci*. 106:19432–19437.
- Song J, Yan HY, Popper AN. Damage and recovery of hair cells in fish canal (but not superficial) neuromasts after gentamicin exposure. *Hear Res*. 1995; 91:63–71. [PubMed: 8647725]
- Steyger PS, Peters SL, Rehling J, Hordichok A, Dai CF. Uptake of gentamicin by bullfrog saccular hair cells in vitro. *J Assoc Res Otolaryngol*. 2003; 4:565–78. [PubMed: 14605921]
- Stuart GW, McMurray JV, Westerfield M. Replication, integration and stable germ-line transmission of foreign sequences injected into early zebrafish embryos. *Development*. 1988; 103(2):403–12. [PubMed: 2852096]
- Stuart GW, Vielkind JR, McMurray JV, Westerfield M. Stable lines of transgenic zebrafish exhibit reproducible patterns of transgene expression. *Development*. 1990; 109(3):577–84. [PubMed: 2401211]
- Tibaldi C, Pazzagli I, Berrettini S, De Vito A. A case of ototoxicity in a patient with metastatic carcinoma of the breast treated with paclitaxel and vinorelbine. *Eur J Cancer*. 1998; 34:1133–1134. [PubMed: 9849468]
- Ton C, Parng C. The use of zebrafish for assessing ototoxic and otoprotective agents. *Hear Res*. 2005; 208:79–88. [PubMed: 16014323]
- Usami S, Takumi Y, Fujita S, Shinkawa H, Hosokawa M. Cell death in the inner ear associated with aging is apoptosis? *Brain Res*. 1997; 747:147–150. [PubMed: 9042539]
- Vandenabeele P, Vanden Berghe T, Festjens N. Caspase inhibitors promote alternative cell death pathways. *Sci STKE*. 2006; 2006:pe44. [PubMed: 17062895]
- Vandenabeele P, Galluzzi L, Vanden Berghe T, Kroemer G. Molecular mechanisms of necroptosis: an ordered cellular explosion. *Nat Rev Mol Cell Biol*. 2010; 11(10):700–14. [PubMed: 20823910]
- Vercammen D, Beyaert R, Denecker G, Goossens V, Van Loo G, Declercq W, Grooten J, Fiers W, Vandenabeele P. Inhibition of caspases increases the sensitivity of L929 cells to necrosis mediated by tumor necrosis factor. *J Exp Med*. 1998; 187:1477–85. [PubMed: 9565639]
- Wang J, Van De Water TR, Bonny C, de Ribaupierre F, Puel JL, Zine A. A peptide inhibitor of c-Jun N-terminal kinase protects against both aminoglycoside and acoustic trauma-induced auditory hair cell death and hearing loss. *J Neurosci*. 2003; 23:8596–607. [PubMed: 13679429]
- 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. [PubMed: 15604295]
- White RM, Cech J, Ratanasirinrawoot S, Lin CY, Rahl PB, Burke CJ, Langdon E, Tomlinson ML, Mosher J, Kaufman C, Chen F, Long HK, Kramer M, Datta S, Neuberger D, Granter S, Young RA, Morrison S, Wheeler GN, Zon LI. DHODH modulates transcriptional elongation in the neural crest and melanoma. *Nature*. 2011; 471:518–22. [PubMed: 21430780]
- Williams JA, Holder N. Cell turnover in neuromasts of zebrafish larvae. *Hear Res*. 2000; 143:171–81. [PubMed: 10771194]
- World Health Organization. Deafness and hearing impairment [Online]. 2010. www.who.int/mediacentre/factsheets/fs300/en/index.html
- Yang WP, Henderson D, Hu BH, Nicotera TM. Quantitative analysis of apoptotic and necrotic outer hair cells after exposure to different levels of continuous noise. *Hear Res*. 2004; 196:69–76. [PubMed: 15464303]
- Yu L, Alva A, Su H, Dutt P, Freundt E, Welsh S, Baehrecke EH, Lenardo MJ. Regulation of an ATG7-beclin 1 program of autophagic cell death by caspase-8. *Science*. 2004; 304:1500–2. [PubMed: 15131264]

- Zhang DW, Shao J, Lin J, Zhang N, Lu BJ, Lin SC, Dong MQ, Han J. RIP3, an energy metabolism regulator that switches TNF-induced cell death from apoptosis to necrosis. *Science*. 2009; 325(5938):332–6. [PubMed: 19498109]
- Zhivotovsky B, Orrenius B. Cell death mechanisms: cross-talk and role in disease. *Exp Cell Res*. 2010; 316(8):1374–83. [PubMed: 20211164]

Summary points

1. The zebrafish lateral line allows rapid and efficient in vivo screening for chemicals that protect and damage hair cells.
2. Hair cell protection screens have yielded candidate protective small molecules and FDA-approved drugs.
3. The lateral line has been used successfully to screen clinically approved drugs and bioactive compounds for hair cell toxicity and may be a useful platform to identify dangerous drugs and drug combinations in humans.

Figure 1.

Schematic of zebrafish lateral line neuromast. Neuromast typically consists of 5–20 hair cells surrounded by mantle of supporting cells. Hair cell apices extend stereocilia and kinocilia into water surrounding fish. (reprinted from Chiu et al., 2008, with permission of Springer Publishing, J Assoc Research Otolaryngol)

Figure 2.

Fluorescence microscopy of zebrafish neuromasts. **A/B** – Neuromasts labeled with YOPRO-1 which labels hair cell nuclei in vivo. In **A**, hair cells are intact with typical lacy chromatin pattern. In **B**, after exposure to neomycin, most hair cells have died with condensed nuclei and nuclear fragments remaining. **C/D** – Immunohistochemistry on fixed tissue using anti-parvalbumin antibody. For hair cell counts, anti-parvalbumin antibody allows easy visualization and quantification of hair cell death in intact (**C**) and damaged (**D**) neuromasts. **E/F** – Texas red-conjugated gentamicin allows assessment of aminoglycoside uptake into YOPRO-1 labeled lateral line hair cells. After 3 minutes of exposure, hair cells are brightly labeled with GTTR (red) in control neuromasts (**E**). However, when treated with some protectants, GTTR uptake is significantly diminished (**F**). Scale bars in **B**, **D**, and **F** = 10 μ m.

Figure 3. Schematic of hair cell toxicity and protection protocols. 5 dpf zebrafish are labeled with fluorescent dye, and then placed into individual wells of a 96-well plate. For hair cell toxicity screens, zebrafish are then treated with library compounds and then directly imaged in 96-well plate. For hair cell protection screens, zebrafish are first treated with library compounds, followed by treatment with hair cell toxin. Zebrafish are then imaged within 96-well plates for evidence of hair cell protection.