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Fish in a Dish: Drug Discovery for Hearing Habilitation

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Abstract

The majority of hearing loss is caused by the permanent loss of inner ear hair cells. The identification of drugs that modulate the susceptibility to hair cell loss or spur their regeneration is often hampered by the difficulties of assaying for such complex phenomena in mammalian models. The zebrafish has emerged as a powerful animal model for chemical screening in many contexts. Several characteristics of the zebrafish, such as its small size and external location of sensory hair cells, uniquely position it as an ideal model organism for the study of hair cell toxicity, protection, and regeneration. We have used this model to screen for drugs that affect each of these aspects of hair cell biology and have identified compounds that affect each of these processes. The identification of such drugs and drug-like compounds holds promise in the future ability to stem hearing loss in the human population.

Keywords

Chemical genetics; hair cell; ototoxicity; zebrafish; lateral line

Hair cells function as sensory receptors of the auditory and vestibular systems in all vertebrates. Progressive loss of hair cells in the cochlear partition of the inner ear through genetic predisposition, aging, environmental noise exposure, and drug toxicity can cause permanent deafness [1-4]. One of the more experimentally tractable causes of hair cell death is exposure to ototoxic agents, which include aminoglycoside antibiotics, such as neomycin and gentamicin, and platinum-based anticancer therapeutics, such as cisplatin [1, 5]. It is estimated that hearing thresholds are elevated in 10-20% of those receiving aminoglycoside antibiotics and as much as 80% in cisplatin-treated patients [3, 6]. However, because of their cost and/or robust effectiveness, these drugs continue to be used while their ototoxic effects often limit drug-dosing paradigms. In addition, new therapeutics are not typically evaluated

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for their ototoxic potential, raising the possibility that drug-induced hearing loss is more prevalent than commonly thought. Drugs that prevent hair cell loss due to ototoxin exposure would therefore offer potential benefit for millions of people.

The first reports of drug-induced hearing loss occurred in the 1940s following administration of the aminoglycoside antibiotic streptomycin to treat tuberculosis [7]. Decades later, we still know relatively little about mechanisms through which these and other compounds exert their ototoxic effects and about how to prevent them. We do know, however, that a number of cellular responses are initiated following aminoglycoside exposure. These include activation of multiple signaling cascades and ROS production, as well as triggers of both necrotic and apoptotic-like cell death mechanisms [8, 9]. Despite these generalities, it is clear that different classes of ototoxins stimulate hair cell death by triggering different combinations of death-inducing pathways. For example, cisplatin induces a number of different cellular responses when compared to aminoglycoside antibiotics [9]. Even drugs within the same general class, (e.g., neomycin and gentamicin) appear to act by different combinations of mechanisms [10]. The initiation of multiple cellular processes following ototoxin exposure raises the possibility that many processes could potentially be targeted alone or in combination to preserve hearing during treatment with ototoxic drugs.

Recent advances in efforts to identify compounds that modulate cellular responses to conditions that impact the survival of inner ear hair cells have revolutionized the search for drugs that provide robust protection or facilitate regeneration. This effort has been facilitated through the use of zebrafish as an experimental platform that has become increasingly used in the study of hearing and vestibular function [11, 12]. In addition to hair cells in the inner ear, zebrafish possess hair cells clustered in structures called neuromasts along the surface of their head and body (Fig. 1) [13]. These groups of hair cells comprise the lateral line system, which senses water movement near the animal and functions in such behaviors as prey detection, predator avoidance, and orientation to current [14-16]. Hair cells of the lateral line share many properties with inner ear hair cells of mammals, including sensitivity to aminoglycoside antibiotics and chemotherapeutic compounds [17-20]. Furthermore, agents that protect hair cells in mammals also protect lateral line hair cells [21], and protectants originally identified in zebrafish have recently been shown to confer similar protective effects against ototoxins in rats [22]. This strongly suggests that cellular pathways activated by ototoxin exposure are conserved between hair cells of the zebrafish lateral line and mammalian inner ear. Unlike mammals, hair cells in zebrafish regenerate following insult [23, 24], offering an additional potential therapeutic dimension to hair cell biology – the potential to reverse hearing loss by studying a system with innate regenerative ability. In this mini-review, we focus on chemical screening using the lateral line of larval zebrafish as a model for mechanosensory hair cell loss, protection, and regeneration. We specifically address these compounds as they pertain to basic research, drug discovery, and repurposing of existing drugs, as well as target identification.

Chemical screens in the zebrafish lateral line

The high fecundity, small size, and optical transparency of larval zebrafish facilitate medium- or high-throughput screening. Larvae are distributed in 96 well plates, administered a wide range of compounds dissolved in water, and assayed for the desired phenotype. Screens of this type have been conducted for a wide range of purposes, from basic biology to drug safety testing [21, 25-33]. Since 2000, our group has used the lateral line in larval zebrafish for drug screening with four primary and intersecting goals: 1) to better understand the pathways underlying ototoxin-induced hair cell death or survival following such challenges [10, 18, 20, 26, 34-38]; 2) to identify current drugs with

unrecognized ototoxic potential [39, 40]; 3) to discover drugs and small molecules that protect hair cells from known ototoxins [26, 41]; and 4) to discover compounds that alter the innate regenerative potential of zebrafish hair cells with the goal of discovering drugs that might trigger hair cell regeneration in mammals [Namdaran et al., submitted] (see Fig. 2). By using zebrafish to screen for compounds that modulate hair cell loss, protection, and regeneration, each phenomenon can be evaluated in its native context – while at the same time offering advantages of a relatively high-throughput, scalable system that is typically associated with cell culture. In addition, the same drug library can be screened multiple times for distinct phenotypic outcomes, allowing us to maximize return on investment.

Identification of compounds with ototoxic potential

The attribution of ototoxic properties to a therapeutic compound typically occurs only after the frequency of anecdotal reports of hearing or vestibular impairment warrants further study in animal models. This is unfortunate from both financial and human suffering points of view. While ototoxicity is most widely recognized for aminoglycosides and platinum-based anticancer drugs, as well as some nonsteroidal anti-inflammatory agents and loop diurectics, the inherent nature of some effective therapeutic drugs to be both cytotoxic and tissue permeant implies that many FDA-approved drugs may be ototoxic to varying degrees. The advent of the zebrafish lateral line system as a valid platform to screen for ototoxic agents has enabled us to efficiently identify novel hair cell toxins from several libraries of FDA-approved drugs and related compounds. Our initial screen of 1,040 compounds in the US Drug Collection library uncovered 21 toxic compounds – seven known ototoxins and 14 additional compounds with previously unrecognized hair cell toxicity [40]. More recently, a screen of 88 anticancer drugs from the NCI FDA-approved oncology drug set confirmed the hair cell toxicity of four known hair cell toxins, further validating the platform's specificity. In addition, we confirmed the hair cell toxicity of four antineoplastic drugs suspected to be potential ototoxins and identified five additional anticancer therapeutics as potential ototoxins [39]. These drugs with formerly unrecognized ototoxicity potential range from mild to severe in their ability to kill lateral line hair cells. For example, we demonstrated that raloxifene is approximately ten times more toxic to lateral line hair cells than cisplatin, whereas dactinomycin is ten times less toxic than cisplatin. While validation in mammalian laboratory model systems will be necessary to confirm their potential for ototoxic effects, these studies highlight the possibility that commonly used therapeutics may contribute to a much greater prevalence of mild to moderate hearing loss within the population than previously thought.

Identification of compounds that protect against hair cell loss

To screen for compounds that protect hair cells against ototoxic drugs, only minor modifications are necessary to our toxicity screens. Our initial screens for hair cell protectants were designed to identify compounds that conferred robust protection to neomycin-treated hair cells [26]. We initially focused on small molecules within the Chembridge Diverset E library. To efficiently screen a sample of 10,960 compounds from the library, compounds were multiplexed five per well and then reassessed individually when protection was observed. Two compounds, PROTO1 and PROTO2, exhibited robust protection of lateral line hair cells over a broad range of neomycin concentrations. Further testing of these compounds in mice revealed that they also protected hair cells in neomycin-treated utricular explants, validating the zebrafish lateral line as a model for discovery of novel otoprotective drugs. Both PROTO1 and PROTO2 are related benzothiophene carboximides, raising the possibility that they may confer their protective effects through action on the same target.

The identification of PROTO1 and 2 highlights the strength of such a screen, as it would be difficult or impossible to select these relatively unknown molecules as candidate protectants of aminoglycoside-mediated hair cell death. The targets of these compounds are unknown at present, however, making it difficult to determine the mechanism of action of these drugs. We are currently using a methodology similar to our original small molecule screen to evaluate the protective benefit of PROTO1 analogues, in hopes of identifying the conserved chemical structure required for otoprotection. In a complementary approach, we have also undertaken a screen of a customized cell death inhibitor library in order to better understand the myriad cell death pathways that are necessary for hair cell death due to specific ototoxins. A small-scale pilot screen conducted in 2005 by an independent research group demonstrated that multiple antioxidants protect lateral line hair cells from cisplatin toxicity, serving as proof-of-concept for basic biology-based screening paradigms [21]. Our current screens point to a complex network of interconnected cell death pathways being activated in response to a single ototoxic drug, and distinct differences in pathway activation following exposure to different aminoglycoside antibiotics.

Repurposing existing FDA-approved drugs

There are numerous economic challenges to *de novo* drug development. FDA approval of new drugs requires approximately 10-12 years and often hundreds of millions of dollars to identify effective compounds with optimal pharmacokinetic profiles [42, 43]. The odds of a newly minted drug being brought to market are approximately 1:5,000 [44], making drug synthesis a far less efficient endeavor than identification of new uses for therapeutics that have already been shown to be safe and effective for human use. Although most drug repurposing success stories are the result of educated guesses pertaining to a compound's mechanism of action on a particular disease state, the paucity of convincing evidence for a singular hair cell death pathway indicates that screening each of the approximately 10,000 FDA-approved therapeutics may be an effective approach in terms of both efficiency and ultimate expense.

We are attempting to undertake such a shortcut from the lab to the clinic. To date, we have screened one such library, the aforementioned US Drug Collection of 1,040 therapeutic compounds [41]. From the Drug Collection we identified seven compounds that protected against neomycin-induced hair cell death, three of which are FDA-approved. Follow-up of these compounds revealed that one compound, the acetyl cholinesterase inhibitor tacrine, was capable of conferring protective effects on mammalian hair cells *in vitro*. We have recently initiated additional screens to identify further FDA-approved therapeutics that confer protection against the hair cell toxicity of multiple aminoglycoside antibiotics and from the neoplastic agent cisplatin.

Blocking ototoxin entry into hair cells: the ultimate protectant?

Perhaps the most appealing way to block hair cell loss associated with ototoxin exposure is by preventing entry into the hair cell before it can induce cellular damage. In the case of aminoglycoside antibiotics, a number of genetic and chemical studies indicate that the major route of entry occurs through mechanotransduction channels [34, 45-53], which are channels of unknown molecular identify that allow hair cells to respond to vibrational (sound) stimuli. In our screen to identify novel hair cell protectants from the US Drug Collection library, we identified four compounds that significantly reduced entry of fluorescently labeled aminoglycoside (gentamicin-conjugated Texas Red) into lateral line hair cells [41] and confer robust protection. This is likely due to impaired mechanotransduction, as uptake of mechanotransduction-dependent vital dyes was also reduced in treated hair cells. Other studies demonstrate that similar protection is achieved by blocking mechanotransduction in

mammals [45]. Although these mechanotransduction inhibitors prevent aminoglycoside entry into hair cells, they do not affect the antibiotic properties of the aminoglycoside itself and thus allow them to remain therapeutically relevant [26]. It is highly likely that a number of other compounds identified in our screens will function, at least in part, by preventing ototoxin entry into hair cells. Compounds that block uptake of non-aminoglycoside ototoxins (e.g., cisplatin) will be of particular interest, although the routes through which these agents enter hair cells remain unknown.

The appeal of preventing aminoglycoside-induced hearing loss by impeding mechanotransduction is tempered by the notion that the very act of fully or partially blocking aminoglycoside entry also renders hair cells themselves nonfunctional. Substantial hearing loss, even if only temporary, would certainly ensue from such a course of action. Because they are thought to operate through the same mechanism as auditory hair cells, mechanotransduction blockers would also be expected to impede the function of vestibular hair cells, the receptors that convey movement and balance information in the vestibular system. The advantages of blocking permanent hearing loss would therefore need to be balanced with any temporary deafness and/or vestibular disorientation that such drugs would inevitably cause. It is unclear how long such drugs retain their ability to block mechanotransduction, raising the possibility that such blockades are irreversible. For example, the long-lasting protective effects following washout of two of the four compounds identified from our Drug Collection library screen, carvedilol and phenoxybenzamine, are indicative of such a long-lasting mechanotransduction blockade.

Screening for compounds that alter hair cell regeneration

While hair cell loss, and the resulting hearing loss, is permanent in humans and other mammals, birds, zebrafish, and other non-mammalian vertebrates possess the remarkable ability to regenerate hair cells and restore sensory function [23, 24]. Hair cells in the larval zebrafish lateral line completely regenerate by 72 hours after toxic neomycin damage [35], making this an ideal system in which to quickly evaluate compounds that alter regenerative potential. This regeneration process is sensitive to small molecule inhibitors such as DAPT, which blocks the Notch signaling pathway [54]. One recent screen of 480 compounds by Moon et al. [55] found that low molecular weight fucoidan enhanced the regenerative capacity of the zebrafish lateral line, apparently by increasing supporting cell proliferation. Our group has screened the US Drug Collection, initially used for our otoprotective screen (see above), and an additional library of 640 drugs from Enzo Life Sciences, for compounds that modulate hair cell regeneration [Namdaran et al., submitted]. This study identified six compounds that inhibited regeneration and two compounds that enhanced hair cell regeneration. Both enhancer compounds are synthetic glucocorticoids, suggesting that modulating steroidal pathways may facilitate other, as yet undetermined, signaling cascades for stimulating hair cell regeneration in the mammalian inner ear.

Intersection between chemical and genetic screens

Zebrafish are particularly amenable for traditional forward genetic screens, with the first large-scale screens conducted to identify genes important for early vertebrate development [56-58] and more recent screens aimed at uncovering genes involved in the development of specific tissues or modification of behavioral phenotypes [59-62]. Our group developed a mutagenesis screen to identify genes that modify hair cell responses to neomycin exposure as a complement to the chemical genetic screens discussed above [26].

Chemical and genetic screens can be combined to permit the identification of both a compound of interest and its putative target(s). This can be particularly useful as any chemical can be potentially pleiotropic in its effects, masking its key target or mechanism of

action in the process of interest. Depending on the experimental protocol, two different scenarios to identify these interactions can be envisioned. In the first, random or selected sets of compounds can be screened for their ability to rescue or modify a particular mutant phenotype. In the second, mutagenesis screening techniques can be used to identify mutants that fail to display a particular phenotype when treated with a compound of interest. Such approaches have been successfully undertaken in zebrafish – in some cases, the drugs identified offer promise in the clinic [25, 30, 63, 64]. Furthermore, in both scenarios, such screens provide valuable information towards identifying the cellular pathways in which a compound of interest acts. These types of combinatorial screening approaches are particularly useful for providing valuable information about the target of compounds discovered in our hair cell chemical screens, such as the otoprotective benzothiophene carboxamides. Conducting such a chemical genetic screen with these mutants and/or compounds offers a promising step to provide protective measures against ototoxin-induced hair cell death in humans, and may provide insight into other forms of hearing loss.

Conclusion

The zebrafish lateral line provides a powerful tool to discover drugs, potential drugs, and genes that affect hearing in the human population. Beyond their translational aspects, molecules that induce or promote hair cell survival possess the power to provide information about the pathways involved in these processes. A better understanding of the pathways involved in ototoxin-induced hair cell death and regeneration will maximize efficiency at which we can predictively design drugs based on their target interactions. These tools have broader impact in their ability to evaluate the similarities and distinctions between drug-induced hair cell death and noise- or age-related hair cell damage.

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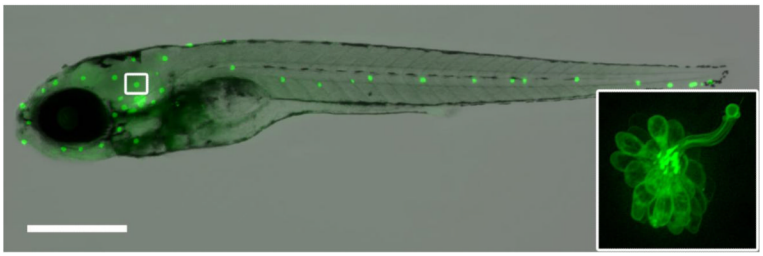

Figure 1.

A transgenic *(brn3c:gfp)* 5 day post-fertilization zebrafish larvae. Sensory hair cells can be observed in green, arrayed around the head and body of the larvae. The inset depicts a higher magnification view of an anterior lateral line neuromast taken from the boxed area. Scale bar = 500 μM, 20 μM in the inset.

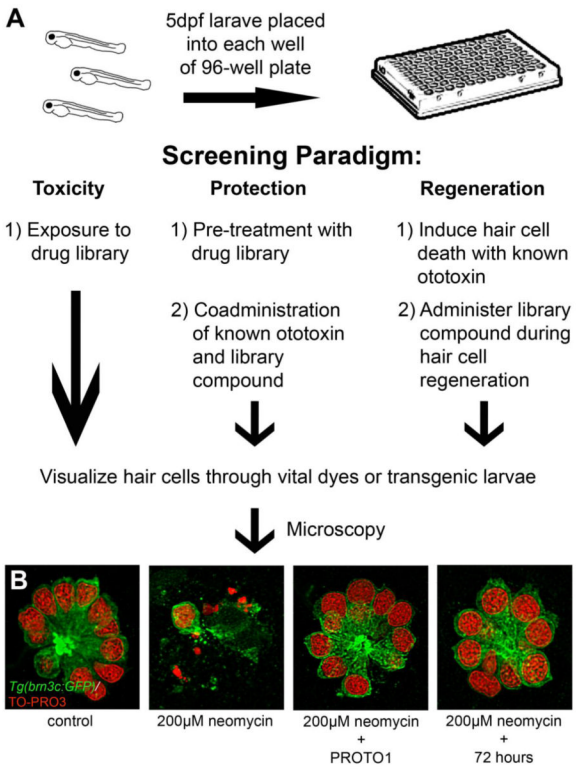

Figure 2.

Library compounds can be administered in distinct screening paradigms to identify compounds that affect different aspects of hair cell biology. A) Following placement in multi-welled plates, 5-day post fertilization zebrafish larvae are exposed to library compounds before, during, or following induced hair cell damage to identify compounds that induce hair cell damage, protect against known ototoxins, or modulate hair cell regeneration, respectively. Following exposure, larvae are treated with vital dyes that selectively stain hair cells, and hair cell numbers can be assayed through basic microscopy techniques. B) Examples of fluorescently labeled hair cells of the zebrafish lateral line. The transgenic marker *Tg(brn3c:gfp)* and vital dye TO-PRO3 selectively label hair cell soma and nuclei, respectively. Hair cell number and morphology can thus be assessed during screening. Although different from the markers used in previous screens, these dyes nonetheless highlight the ease at which hair cells can be visualized *in vivo*.