Mechanism of ORC-13661 hair cell protection

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Background

Hair cells are specialized mechanosensory cells that are essential for normal functioning auditory and balance systems. Within the cochlea and vestibular organs hair cells translate mechanical energy from sound waves and head movements into action potentials, respectively. Hair cells interpret kinetic energy with the mechanoelectrical transduction (MET) apparatus. MET channels confer mechanosensitivity in hair cells, but also serve as an entry route for ototoxins.

Aminoglycoside antibiotics that are most frequently employed to treat multidrug resistant *Mycobacterium tuberculosis* infections, gram-negative sepsis, and resistant strains of *Pseudomonas aeruginosa* in patients with cystic fibrosis. Nephrotoxicity is commonly anticipated with use of these drugs; however, ototoxicity is often underappreciated by clinicians and there are no reliable strategies to screen for or prevent permanent hearing loss.

ORC-13661 is a thiophene-urea-carboxamide small molecule that demonstrates robust hearing protection against aminoglycoside toxicity (Fig. 1) and is currently under investigation in clinical trials (Chowdhury et al., 2018; Kitcher et al., 2019). Despite the advances that have been made with this novel therapeutic, we still do not fully understand its mechanism of action. The goals of my investigations are to I) identify the target of ORC-13661 and II) test the hypothesis that ORC-13661 blocks MET-dependent uptake of aminoglycosides in living mammalian hair cells.

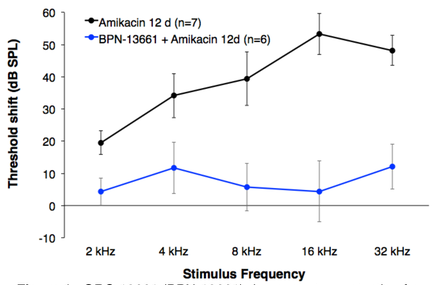

Figure 1. ORC-13661 (BPN-13661) demonstrates protection from amikacin-induced hearing loss in rats. ABR threshold shifts were calculated in rats treated with amikacin (black) and ORC-13661 + amikacin (blue). (Chowdury et al., 2018)

Here, preliminary data using newly synthesized analogues of ORC-13661 demonstrates that photoreactive analogues can provide irreversible hair cell protection. We further show evidence that bifunctional analogues with photoreactive groups and biotin tags can be used to label intracellular targets in hair cells and pull-down intracellular proteins. These results will guide follow up proteomics studies to identify binding partners of ORC-13661. Finally, we show data from a pilot study in neonatal mice demonstrating that hair cells take up fluorescently-labeled aminoglycosides. These results will be used as the foundation to test our hypothesis and ORC-13661 hair cell protection is due to decreased MET-dependent uptake of ototoxins.

Methods

Chemical toolbox of ORC-13661 analogues:
A library of ORC-13661 of photoreactive and biotinylated ORC-13661 analogues was synthesized. Benzophenone moieties are photoreactive, and upon exposure to ultraviolet (UV) light form a free radical capable of forming a covalent bond with nearby organic molecules. Biotin functional groups were also added to compounds to allow for streptavidin affinity labeling and pull-downs. Prior to use in these studies, all compounds were validated in hair cell protection assays to ensure that the added functional groups did not abolish hair cell protection from aminoglycosides.

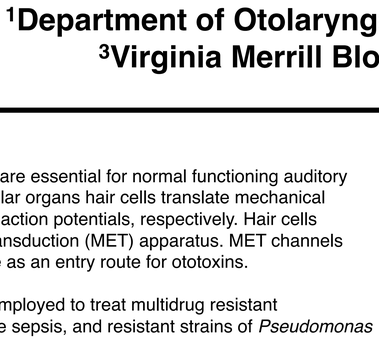

Figure 2. ORC-13661 and examples of photoreactive and biotinylated analogues. Blue boxes highlight photoreactive benzophenone moieties and the red box indicates a biotin tag for labeling and pull-down experiments.

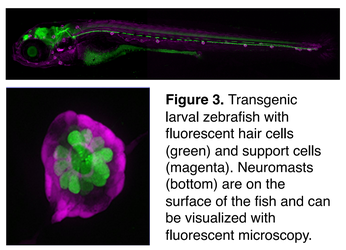

Figure 3. Transgenic larval zebrafish with fluorescent hair cells (green) and support cells (magenta). Neuromasts (bottom) are on the surface of the fish and can be visualized with fluorescent microscopy.

Zebrafish neuromast hair cell system:
The lateral line sensory system found in aquatic vertebrates contains mechanosensory hair cells structurally and genetically similar to mammalian hair cells. Five-day post-fertilization live zebrafish were treated with ORC-13361 analogues ± UV irradiation. For protection studies, fish were exposed to neomycin, fixed, and hair cells were labeled with anti-parvalbumin for quantification. For localization studies, fish were fixed, hair cells were labeled with phalloidin, and biotinylated compounds were labeled with streptavidin or anti-biotin antibodies.

Mammalian hair cell model system:
Neonatal hair cells from C57BL/6J (Black 6) mice were used to evaluate localization of ORC-13661 analogues and uptake of fluorescently-labeled aminoglycosides. For localization studies, P2 pups were sacrificed and the organ of Corti was dissected and cultured in DMEM/F12 + FBS overnight. Organotypic cultures were then treated with analogues, fixed, and labeled with phalloidin to identify hair cells and streptavidin. Whole mount specimens were then examined under high-resolution confocal microscopy. For uptake experiments, Texas Red-labeled gentamicin was synthesized and purified (G418-TR). Live P5 pups received subcutaneous injections with vehicle or 2 mg/kg G418-TR. Mice were incubated for three hours before sacrifice. Otic capsules were dissected and fixed. Cochlear sensory epithelia was then dissected, labeled with anti-myosin-VIIa (hair cells) and whole mount specimens were analyzed with confocal microscopy.

Preliminary Results

Photoreactive ORC-13661 analogue demonstrates irreversible hair cell protection in zebrafish after UV irradiation

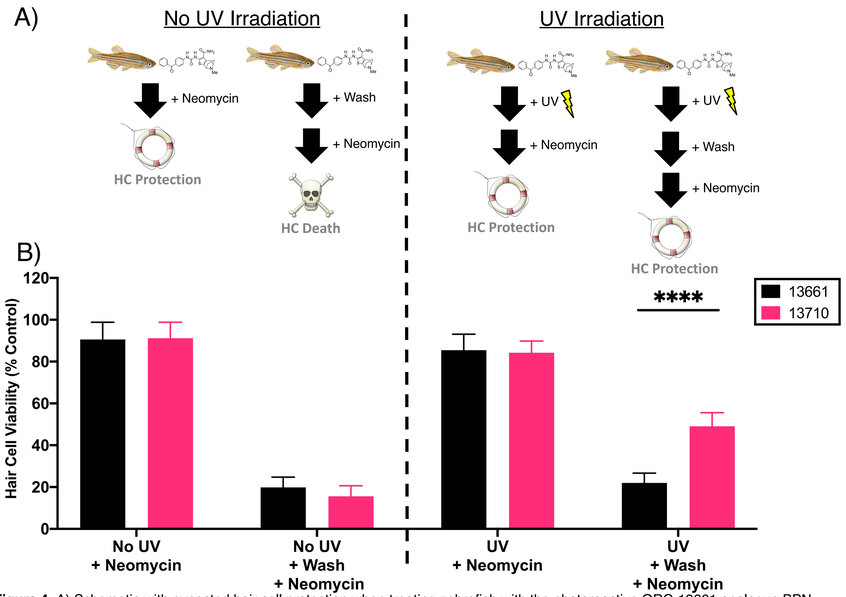

Figure 4. A) Schematic with expected hair cell protection when treating zebrafish with the photoreactive ORC-13661 analogue BPN-13710 ± UV irradiation. Without UV irradiation, 13710 protects against neomycin, however this can be reversed by washing away the compound. With UV irradiation, photoreactive analogues protect against neomycin before and after after a washout step, suggesting the formation of an irreversible covalent bond. B) Zebrafish hair cell survival when treated with ORC-13661 (2.5 μM) or BPN-13710 (50 μM) and exposed to neomycin (200 μM). UV Photoactivation results in partial irreversible hair cell protection for fish treated with BPN-13710. Data plotted are mean with standard deviation error bars. P values obtained from two-way ANOVA with post-hoc Sidaks multiple comparisons test. *****P < 0.0001*

Photoreactive and biotinylated ORC-13661 analogues can be used to label hair cells and pull-down proteins

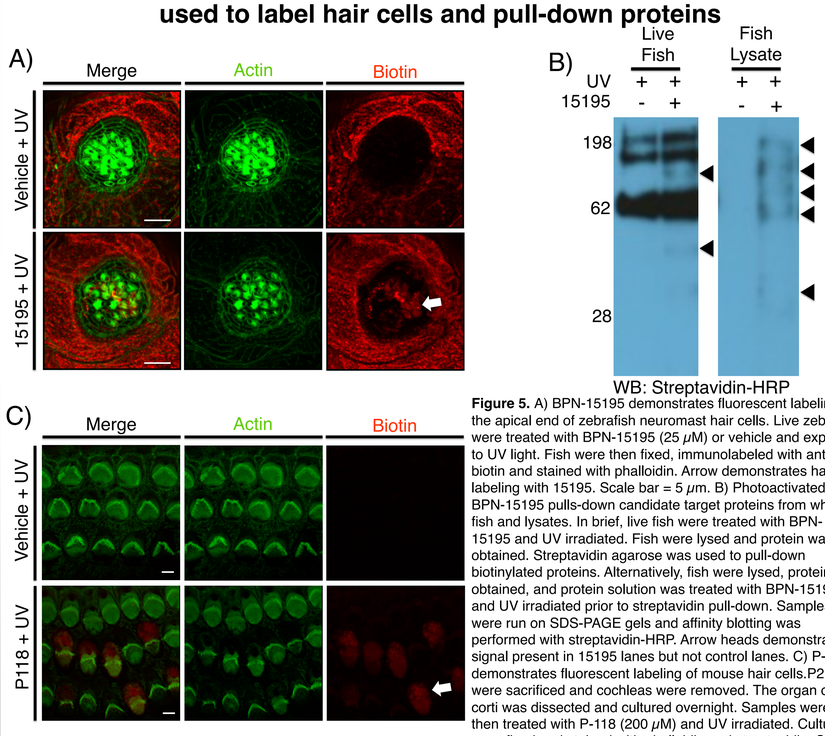

Figure 5. A) BPN-15195 demonstrates fluorescent labeling of the apical end of zebrafish neuromast hair cells. Live zebrafish were treated with BPN-15195 (25 μM) or vehicle and exposed to UV light. Fish were then fixed, immunolabeled with anti-biotin and stained with phalloidin. Arrow demonstrates hair cell labeling with 15195. Scale bar = 5 μm. B) Photoactivated BPN-15195 pulls-down candidate target proteins from whole fish and lysates. In brief, live fish were treated with BPN-15195 and UV irradiated. Fish were lysed and protein was obtained. Streptavidin agarose was used to pull-down biotinylated proteins. Alternatively, fish were lysed, protein was obtained, and protein solution was treated with BPN-15195 and UV irradiated prior to streptavidin pull-down. Samples were run on SDS-PAGE gels and affinity blotting was performed with streptavidin-HRP. Arrow heads demonstrate signal present in 15195 lanes but not control lanes. C) P-118 demonstrates fluorescent labeling of mouse hair cells.P2 mice were sacrificed and cochleas were removed. The organ of corti was dissected and cultured overnight. Samples were then treated with P-118 (200 μM) and UV irradiated. Cultures were fixed and stained with phalloidin and streptavidin. Scale bar = 5 μm. Arrow demonstrates hair cell labeling.

In vivo uptake of gentamicin labeled with Texas Red (G418-TR) in mammalian hair cells

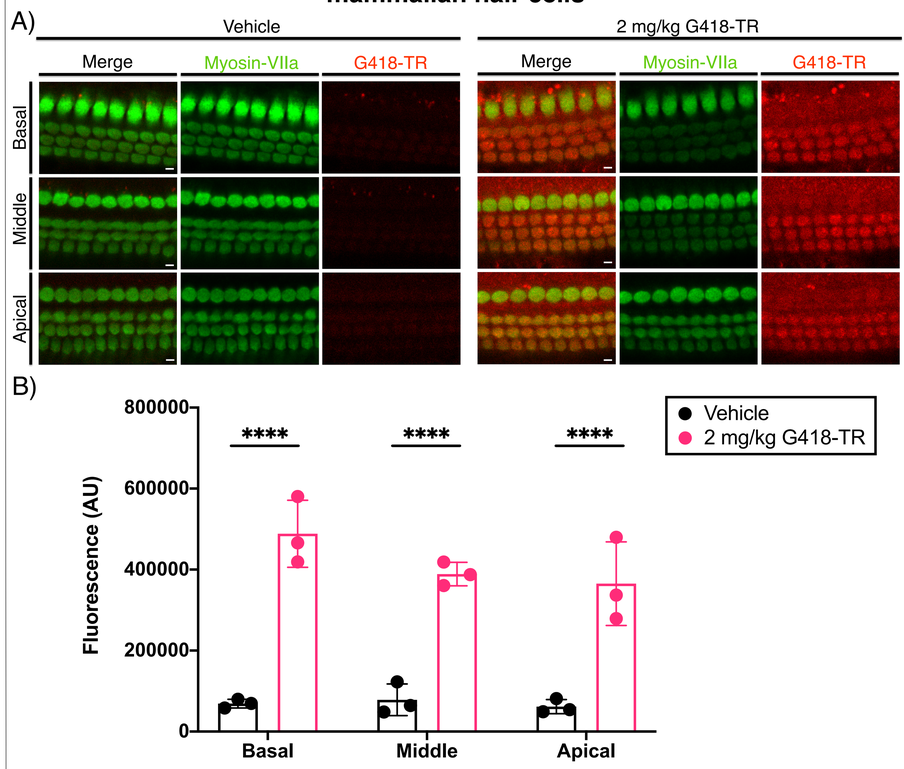

Figure 6. A) *In vivo* mouse hair cell uptake of gentamicin labeled with fluorescent Texas Red (G418-TR). P5 Black 6 mice were treated with saline vehicle or 2 mg/kg G418-TR by subcutaneous injection and incubated for 3 hours. Mice were then sacrificed, otic capsules were dissected and fixed, and cochlear sensory epithelium was removed. Hair cells were immunolabeled with anti-MyosinVIIa antibodies. High resolution confocal microscopy was performed, and images were collected from the basal, middle, and apical turns of the cochlea. Scale bar = 5 μm. B) Quantification of G418-TR signal shows uptake in basal, middle, and apical turns of the cochlea. In brief, fluorescent images were obtained in the z-plane capturing hair cell bodies between the cuticular plate and nucleus. The myosin-VIIa green fluorescent channel was used generate a hair cell specific region of interest, and the integrated density of the red fluorescent signal was calculated for each region of interest. For both control and G418-TR treated animals, n=2 animals and 3 ears. Means with standard deviation error bars are plotted. *****P < 0.0001* P values obtained from two-way ANOVA with post-hoc Sidaks multiple comparisons test.

Preliminary Conclusions

- Photoreactive analogues of ORC-13661 irreversibly protect hair cells from aminoglycosides after UV photoactivation
- Photoreactive and biotinylated analogues of ORC-13661 label intracellular targets in zebrafish and mammalian hair cells
- Photoreactive and biotinylated analogues of ORC-13661 pull-down candidate target proteins from zebrafish
- Neonatal mouse hair cells show uptake and accumulation of fluorescently labeled gentamicin after *in vivo* systemic treatment

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