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A phosphatase and protease library screen to elucidate aminoglycoside-mediated murine sensorineural hair cell damage processes

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A phosphatase and protease library screen to elucidate aminoglycoside-mediated murine sensorineural hair cell damage processes

A thesis submitted in partial satisfaction of the requirements for the degree Master of Science

in

Biology

by

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2020

The Thesis of Taylor Ellyse Wyrick is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Co-chair

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Chair

University of California San Diego

2020

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LIST OF SYMBOLS

HC – hair cell

AG – Aminoglycoside Antibiotics

OC – Organ of Corti

GFP – Green Fluorescent Proteins

ROS – Reactive Oxygen Species

GM – Gentamicin

DMSO – dimethyl sulfoxide

DMEM - Dulbecco's Modified Eagle Media

DPBS – Dulbecco's Phosphate Buffered Saline

FBS – Fetal Bovine Serum

NA(P)DH - nicotinamide adenine dinucleotide

MAPK – mitogen-activate protein kinase

ERK – extracellular-signal-regulated kinase

FGFR – fibroblast growth factor

IGFR – insulin-like growth factor

PI3k – phosphatidylinositol 3-kinase

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ACKNOWLEDGEMENTS

I am so grateful for Dr. Ryan's guidance, support and wisdom along this journey. Dr. Clara Draf, Kwang Pak, and Eduardo Chavez I so appreciate your teaching and support from the beginning of technical training all the way through my project and beyond. Dr. Arwa Kurabi your insight and guidance has been instrumental throughout this process. I am incredibly fortunate to have found this group you were all integral to my research experience and learning.

ABSTRACT OF THE THESIS

A phosphatase and protease library screen to elucidate aminoglycoside-mediated murine sensorineural hair cell damage processes

by

Taylor Ellyse Wyrick

Master of Science in Biology

University of California San Diego, 2020

Professor Allen F. Ryan, Chair

Professor Cory M. Root, Co-Chair

Ototoxicity has implications on hearing loss and vestibular system function due to its damaging effects on the sensorineural cells of the inner ear. These cells have variable sensitivity to ototoxic compounds and limited ability to regenerate making hearing loss due to their death permanent. It is vital to find neuroprotective compounds to develop as drug candidates to prevent

damage to these cells, thus prevent hearing loss. An in vitro library screening-assay of protease and phosphatase inhibitors activating a number of signaling pathways and downstream effectors contributing to hair cell survival producing possible new drug candidates for future testing in developing treatment techniques. The 6 compounds with effective dose-dependent results from this experiment can help clarify the processes and possible new target areas for the treatment of sensorineural hearing loss in mammals with further research.

I. INTRODUCTION

1. Ototoxicity

Ototoxicity refers to the side effects of a drug or pharmaceutical treatment to be specifically toxic to the cochlea, vestibular cochlear nerve, and vestibular system sensory organs inner ear. This is a serious side effect of chemotherapy drugs like cisplatin and aminoglycoside antibiotics like gentamicin which are used to treat life-threatening diseases and infections for patients of all ages. Etiologies of hearing loss include noise-induced hearing loss, drug treatments, middle and inner-ear infection or inflammation due to immune response.

Hearing loss is present in approximately half of patients treated with these life-saving medications as well as in elderly patients who develop the most common form of this impediment to daily communication with presbycusis or age-related hearing loss. Pediatric patients treated with chemotherapy are exceptionally vulnerable to ototoxic effects throughout their lives as sensorineural hearing loss will affect their ability to learn language and subsequently alter their social development (Kent).

Vestibular disorders contribute to the likelihood of falls that can cause additional injuries which is the leading cause of death in elderly patients by altering balance and spatial awareness. Dysfunction of the vestibular system can lead to other can lead to changes in hearing, vertigo, and dizziness. The effects of these medications on sensorineural damage and can be reversible or permanent. These conditions can be exacerbated with chronic noise exposure and confounding diagnoses of diabetes and high blood pressure.

2. Chemotherapy and cisplatin mediated hearing loss

Small molecules containing platinum like cisplatin are strikingly effective anticancer treatments. Cancer treatment widely uses chemotherapy drugs to attempt to slow tumor growth

and give the immune system additional time to attack tumors. However, these treatments can have adverse effects on hair cells, the sensorineural cells of the inner ear, and semicircular canals, the three organs of the vestibular system. Cisplatin mediated ototoxicity perturbs the naturally low intracellular chloride concentrations after diffusion or transportation into the cell and becoming hydrated. Its hydrated, dechlorinated form crosslinks with nuclear and mitochondrial DNA to inhibit cancer cell proliferation to slow tumor growth. The complete mechanism for cisplatin mediated ototoxicity is not fully understood and is an active area of research.

Chemotherapy drugs can activate death receptor pathways and signal the mitochondria to release reactive oxygen species (ROS) that contribute to ototoxicity. These ototoxic agents can be cochleotoxic affecting the inner ear hair cells to produce permanent hearing loss or vestibulotoxic affecting balance and spatial awareness of the body. It is vital to understand the molecular mechanisms that could be causing sensorineural cell damage and loss in the inner ear to create better protocols for treatments with these drugs. Drug candidates are a significant area of research to determine neuroprotective compounds that act on sensorineural cells of the inner ear known as hair cells. Current treatments for ototoxic hearing loss utilize system-wide steroid administration. It is imperative that we find compounds that are able to protect against and lessen the side effects for cisplatin-induced ototoxicity.

3. Aminoglycoside antibiotics and gentamicin mediated hearing loss

Aminoglycoside antibiotics are cationic molecules that prevent bacterial growth by inhibiting ribosomal protein synthesis by binding to 30S subunit and are commonly known as broad-spectrum antibiotics in medical settings (Lanvers-Kaminsky). Antibiotic molecules can access HCs through non-selective cation mechanotransduction channels residing on the apical bundle of

the stereocilia. The electrochemical gradient holding these sensorineural cells at their resting membrane potential creates a driving force to pull the molecule through the ion pore of channels like NMDA expressed on their membranes (Kent). Gentamicin, an aminoglycoside antibiotic, contributes to the overproduction of ROS in HCs which is one of the known mechanisms responsible for HC degradation. Exposure to gentamicin is toxic to outer row HCs and prolonged exposure leads to damage and loss of HCs in the three inner rows.

Aminoglycoside antibiotics contribute to pro-apoptotic signaling through inhibition of caspase 8 and 9 as well as contributing the phosphorylation of c-Jun increasing the activity of the JNK pathway. These processes function as subsequent means of HC degradation and death due to antibiotic exposure following generation of ROS and preceding increased intracellular calcium. Finally, cytochrome C is unleashed in the cytosol to degrade proteins before apoptosis can occur. For the purpose of this research, gentamicin was the antibiotic used to inflict damage on the HC in order to test the neuroprotective or neurotoxic effects of each inhibitor compound as it prevents or contributes to either of these processes as well as any other undiscovered means of HC degradation.

4. Importance of hair cells in hearing loss

Hearing is one of the major senses for mammals to communicate and perceive cues from the surrounding environment. A healthy auditory system's function and signal transmission from the cochlea is an integral part of development and social learning for mammals. Hair cells (HC) are the discrete units of sensory stimulus detection in the auditory nervous system. They are responsible for translating vibrational frequencies detected in the oscillation of various parts of the cochlea. The mammalian cochlea contains two categories for hair cells, inner hair cells which relay acoustic input, and outer hair cells that amplify the sound-evoked vibrations. The cochlea

holds the auditory sensory organ, known as the organ of Corti. Each of these mechanosensitive hair cells lining the organ of Corti is equipped with stereocilia physically linked by tip-link proteins that serve as sensory receptors responding to perturbations in the endolymph these cells are suspended in.

These cells directly detect sensory stimulus and project to spiral ganglion which synapse onto the vestibulocochlear nerve to pass signals onto the primary auditory cortex for processing. Outer row HCs respond to higher frequencies than the 3 rows of their inner ear counterparts. Inner ear hair cells are the most susceptible to ototoxic drug exposure with the outer row of HCs proving more vulnerable than the three rows of inner HCs. Genetic susceptibility to aminoglycoside-mediated hearing loss varies. Sensitivity to ototoxic compound exposure also varies increasingly from apex to base of the cochlea. The molecular mechanisms restraining HC survival are not well-defined and remains an active research focus.

HCs do not have the capacity to regenerate as neural tissues are post-mitotic limiting in their ability to repopulate in order to preserve function. Thus, HC degradation creates permanent, irreversible hearing loss that requires additional investigation into neuroprotective compounds to preserve hearing. Scientists have extensively studied the effects of reactive oxygen species produced by the mitochondria of HCs and their associations with the early stages of ototoxic damage.

5. Generation of Reactive Oxygen Species

Oxidative phosphorylation utilizes the electron transport to generate adenosine triphosphate on the cellular level in the mitochondria utilizing tightly regulated reactive oxygen species, but the overproduction of ROS is one cause of HC degradation. The generation of ROS species presages any discernable damage to HCs by up to 24 hours. Molecular mechanisms for HC

damage are not fully understood, but research suggests ROS contribute to heightened lipid peroxidation, calcium influx, and inflammatory response in HCs leading to increased oxidative stress and activation of apoptosis signaling. Hydrogen peroxide and superoxide production leads to an increase in manganese superoxide dismutase in HCs leading to which may be a result of altered HC metabolism in their mitochondria. These changes have also been associated with ototoxic drug exposure (Hirose, Kim).

Metabolism dysfunction generating and accumulating ROS can be contributed to by treatment with aminoglycoside antibiotics and chemotherapy. Aminoglycoside antibiotics alter nicotinamide adenine dinucleotide production which is measured to by consumption of NAD(P)H dehydrogenase in the electron transport chain. These changes in NAD(P)H concentrations in both outer and inner HCs are characteristic of known mechanisms with aminoglycoside antibiotics which can be seen far before the release of ROS from the mitochondria. Additional research has been conducted to screen compounds that could contribute to ototoxicity or accelerate its effects on cell degradation through downstream MAPK, apoptosis and necroptotic stress signaling.

Given extensive evidence linking ROS to HC damage, and the extensive network of cellular events linked to HC loss, we choose to focus on screening compounds that inhibit general degradation (proteases) and kinase dephosphorylation (phosphatases). The outer HC are only existing in mammals and are the most sensitive to damage. For this reason, we used microexplants of the murine cochlea to directly screen and evaluate many different potential HC protectants using the same ototoxic damage stimulus. This would allow us to identify novel compounds that protect or damage the outer and inner HCs from gentamicin (GM) damage *in*

vitro, to compare levels of protection, and the future to extend these findings to noise damage *in vivo*.

II. MATERIALS AND METHODS

1. Animals

Mice utilized in this experiment were *pou4f3*/GFP line neonates from 3 to 5 days old. These animals were bred in the Dr. Allen F. Ryan laboratory for research use. All experiments were approved by the Institutional Animal Care and Use Committee of the Veterans Affairs Medical Center in San Diego, California and performed according to National Institute of Health guidelines for care and use of laboratory animals.

2. Phosphatase & Protease Inhibitor Libraries

Libraries were purchased from Enzo Life Sciences Screen-Well Phosphatase and Protease Inhibitor libraries. The phosphatase and protease inhibitor compounds used consisted of 86 inhibitory compounds total, 33 phosphatase and 53 protease inhibitors. Compounds were dissolved in DMSO at 10 mM concentrations in 500 µl deep well plates.

3. Dissection & microexplant preparation

Neonatal mouse pups expressing GFP only in HC were sacrificed, and their cochleae were exposed by removing its outer capsule and dissected out of the skull under a microscope in DPBS before conducting further microdissection of the cochlear layers to remove the stria vascularis and organ of Corti. Each organ of Corti was micro-dissected, the apical and lower part of the basal turns were discarded, as they have been found to be relatively invulnerable to aminoglycoside toxicity. The middle turn tissue was segmented into microexplants after being treated with thermolysine to help separate the sensory epithelium. Each microexplant, consisting of about 20 inner HCs and associated outer HCs, was transferred in fluid with a pipet into an

individual well of a 96-well plate with DMSO and cell culture media consisting of 9.22 ml DMEM, 250 µl of HEPES, 30 units/ml penicillin and 5% FBS, sustained in a humidified tissue culture incubator at 37 degrees Celsius with a 5% concentration of carbon dioxide.

4. *In vitro* Phosphatase and Protease Library Screening

These three aliquots of pharmaceutical inhibitor compounds were diluted to a concentration of 0.1% from the original stock solutions to produce 10 µM, 100 µM, and 1000 µM doses. The triplet microexplant samples were each treated with 2 µl of 200 µM of gentamicin and varying dosages of 10 µM, 100 µM and 1000 µM of each of the 86 phosphatase or protease inhibitor compound after 24 hours of cell tissue media exposure. Three wells contained only cell tissue media to serve as negative controls and three wells served as positive controls treated with only gentamicin and cell culture media. A final triplet of controls was exposed to only the most concentrated dose of the test compound and cell culture media to evaluate the effect of each compound alone. The samples were maintained in culture for 24 hours before being treated with gentamicin plus additional doses of each inhibitor and observed for 72 hours after gentamicin exposure. Samples were photographed with a fluorescent microscope every 24 hours following transfer.

5. Data Analysis and Statistics

Raw data from these screen studies were counted manually and normalized by concentration with respect to control experiments with microexplants exposed to only cell culture media and cell culture media plus gentamicin for each experiment. The normalized values were tested for significance first with a Kruskal-Wallis test for preliminary significance and significant compounds were subsequently tested with a two-way Mann-Whitney test. The effective compounds which passed both the Kruskal-Wallis and Mann-Whitney tests and their effects are

enumerated below. The implications of their effects will be discussed further.

III. RESULTS

Out of 86 compounds, a small group of compounds had significant, dose-dependent protective effect while others exhibited enhanced hair cell loss in the absence of gentamicin treatment showing characteristics of sensorineural toxicity. Microexplants treated with only cell culture media showed minimal degradation and loss on days one through three with more significant cell death on day 4, whereas the microexplants treated with 200μM gentamicin show HC loss in the first day after exposure and more severe losses with prolonged exposure on days 2 through 4. The effective pharmacological compounds were 3compounds from the phosphatase inhibitor library and 3 compounds from the protease inhibitor library.

1. Phosphatase Compounds

Tyrosine Phosphatase inhibitor RK-682

RK-682 is a potent, specific inhibitor of tyrosine phosphatase that act on tyrosine kinase. These proteins can dimerize and function as receptors in neural circuits in the human brain (Heneberg). Tyrosine kinase enzymes might be effective pharmaceutical targets for a number of diseases and disorders including hearing loss. RK-682 showed protective effects at all three experimental doses in the presence of gentamicin and showed no change in HC survival at the 1000 μM ($p<0.05$) dose alone (Figure 2).

Mammalian alkaline phosphatase (ALP) inhibitor LevamisoleHCl

LevamisoleHCl functions as a Mammalian alkaline phosphatase inhibitor that acts on ALP and results in alteration of the downstream signaling cascade contributing to HC degradation. ALP has been implicated in Alzheimer's research and bone growth, but little is known about its physiological function in the inner ear. LevamisoleHCl showed protective

effects at the 100 µM ($p<0.05$) and 1000 µM ($p<0.05$) doses in the presence of gentamicin, while the 1000 µM dose alone did not have any significant effect on HC survival (Figure 4). The inhibitor is responsible for preventing the alteration of the phosphorylation state of ALP leading to prolonged activation of the enzyme and likely amplified downstream signal which suggests that ALP activity may be implicated in HC survival.

Tyrosine Phosphatase inhibitor RWJ-60475

RWJ-60475 is a cell-permeable acetoxymethyl ester with the enzymes target of tyrosine phosphatase family which is essential in immune cell antigen receptor signal transduction (Hermiston). Specific forms of tyrosine kinase like CD45 are implicated in the process of translating environmental stimulus to signals for cellular response which can be modulated by interactions with the TCR and lymphocyte phosphatase-associated phosphoprotein whose function is unknown (Hermiston). This inhibitor can cause alterations in downstream Src kinase activity by altering its phosphorylation state (Roskoski). When inhibited these cells activate antigen receptor signaling leading to an increased immune response that could contribute to the advanced degradation of HCs via phagocytosis during apoptotic cell death. The physiological implications of these interactions are not clear, and these enzymes may be further modulated by the accumulation of ROS during HC degradation by an unknown mechanism (Hermiston). RWJ-60475 showed to be protective to HCs at the 10 µM ($p<0.05$) and 100 µM ($p<0.05$) experimental doses (Figure 3).

2. Protease Compounds

Matrix Metalloproteinase-13 (MMP-13) inhibitor CL-82198HCl

MMP-13 is responsible for maintaining bone health in the murine ossicles and is expressed with cathepsin K in the sensorineural cells of the cochlea to preserve normal hearing

(Jáuregui). It is activated by N-terminus removal creating a Zinc-dependent endopeptidase enzyme to cleave collagen to promote healing. The compound maintains the inactive state of MMP-13 by keeping the Zinc-dependent enzyme in its inactive state, thus rendering it unable to function and signal downstream apoptosis and degradation mechanisms. CL-82198HCl exhibited protective effects at all three experimental doses ($p<0.05$) with gentamicin exposure (Figure 5).

Matrix Metalloprotease (MMP) inhibitor Epillocatechin gallate

Epillocatechin gallate is a phenolic antioxidant found in green tea that acts as an inhibitor targeting MMP endopeptidases (Schwander). It acts on the MMP family is implicated in cochlear synapse restructuring after noise-induced damage alters the HCs as well as extracellular matrix regeneration (Freidrich). The MMP family proteases have a number of effects throughout the body with multiple forms, but MMP2 and MMP9 are expressed in the murine cochlea (Li). Epillocatechin gallate had significant protective effects on HC survival in the presence of gentamicin at all three experimental doses with no effect on HC survival at the 1000 µM dose of the compound alone ($p<0.05$) (Figure 7).

Prolyl endopeptidase inhibitor Z-Prolyl-prolinal

Prolyl endopeptidase is able to quickly breakdown proteins containing proline including hormones (Odaka). It is found in the brain and microglia and modulate degradation signaling with its activation in the nervous system by activating microglia (Natunen). Z-Prolyl-prolinal promotes the breakdown of proline containing proteins by maintaining the active state of Prolyl endopeptidase and exhibited protective effects at 100 µM ($p<0.05$) and 1000 µM ($p<0.05$) doses with gentamicin treatment while the 10 µM ($p<0.05$) dose had no significant effect on HC degradation.

IV. DISCUSSION

Our results are informative regarding the role of phosphor-kinase signaling in hair cell damage mechanisms. We evaluated a library of 33 phosphatase inhibitors, which would inhibit the dephosphorylation of kinases, leaving them in their active state, as well as other molecules regulated by phosphorylation. These would be expected to enhance damage and/or survival signaling, depending upon the phosphatase targets. Only 3 of the 33 phosphatase inhibitor compounds showed significant dose dependent effects on HC degradation or survival.

Protease inhibitors inhibit the general degradation of proteins in tissue which is known to be triggered by noise exposure. After evaluating a library of 53 protease inhibitors, only 3 had significant effect in a dose dependent manner. Many of these enzymes are expressed and localized with other similar proteins that have similar cellular function and these inhibitors may have additional effects that were not detected in the library screen and would be better clarified with further experimentation. Testing additional compounds with different binding affinities and chemical properties may cause different effects on downstream signaling leading to a significant deviation in HC survival or damage. Further experimentation with these compounds to refine the effective dose would minimize side reactions and additional binding to enzymes with lesser binding affinities. The dose-dependent nature of these reactions is vital to the function of the protease and phosphatase enzyme as it is responsible for its ability to correctly and preferably bind to its individual substrate(s).

Previous studies, the Ras signaling pathway includes MAPK and ERK enzymes that are implicated in HC survival signaling and their downstream p38 and JNK signals typically activate cell death pathways. Interplay between the signals in HCs from this pathway and a number of

other inputs from various enzymatic activity is crucial in preventing or accelerating cell damage (Battaglia). Gentamicin might directly activate this pathway with an unknown receptor or may indirectly contribute to HC death by modulating the Ras-GTPase activity. The accumulation of ROS may contribute to the stimulation of Ras resulting increased cell survival by downstream activation of ERK. The activation of cell survival signaling outweigh those of the pro-degradation pathway in the case of MAPK activation leading to HC survival by altering farnesyl-transferase activity (Battaglia). The alteration in signaling can also activate PI3K through multiple inputs.

Tyrosine phosphatases could cause changes in PI3K -AKT signaling through the stimulation of FGFR and IGFR due to the prolonged activation of tyrosine kinases. This sustained activity could lead to additional survival signaling that is prolonging HC survival. Inhibiting these phosphatases may lead to prolonged protein synthesis by activating the downstream mTOR complex to maintain cell function.

These MMP endopeptidases have also been implicated in HC survival or damage showing short-term protective effects when administered before noise exposure (Hu). The activity of these enzymes is responsible for altering nuclear expression and rescuing HCs from acoustic injury immediately after exposure but can potentiate damage leading to increased HC degradation when noise exposure is sustained. CL-812198HCl and Epillocatechin gallate prevent the MMP from functioning in healthy cells suggesting that these enzymes may be implicated in HC survival signaling through an unknown mechanism (Hu). The short-term effects of MMPs are consistent with the inhibitor compounds in this study prolonging HC survival *in vitro.*

Effects of prolyl endopeptidase enzyme inhibition may contribute to HC survival signaling by preventing the degradation of proline rich adaptor proteins in the stereocilia,

particularly hair bundle proteins harmonin-b and whlrlin (Schwander). Alterations in these proteins effect the animals hearing directly with mutations resulting in a deaf phenotype. HC survival might be due to preservation of these proline-rich domains mediated by inhibiting prolyl endopeptidases with Z-prolyl prolinal.

It would be useful for methodology of these screening assays create some sort of consistent data processing when it comes down to normalizing GFP expression between cells. We must consider the unknown processes that are confounding HC survival. Existing cell counting procedures are largely manual and give rise to a considerable amount of human error. It could be more precise to create an automated system or software set for specific cell size dimensions and qualifications for a range of quantified fluorescence. This would prevent additional variation in counts and maintain consistency across the standards for inclusion in survival percentages. There is further experimentation to be conducted on the process of mitochondrial degradation, ROS generation, and release of these molecules with Cytochrome c into HCs. Characterizing the expression of these protease and phosphatase enzymes in the HCs before attempting to determine the function of each of these enzymes in the context of HC degradation.

APPENDIX

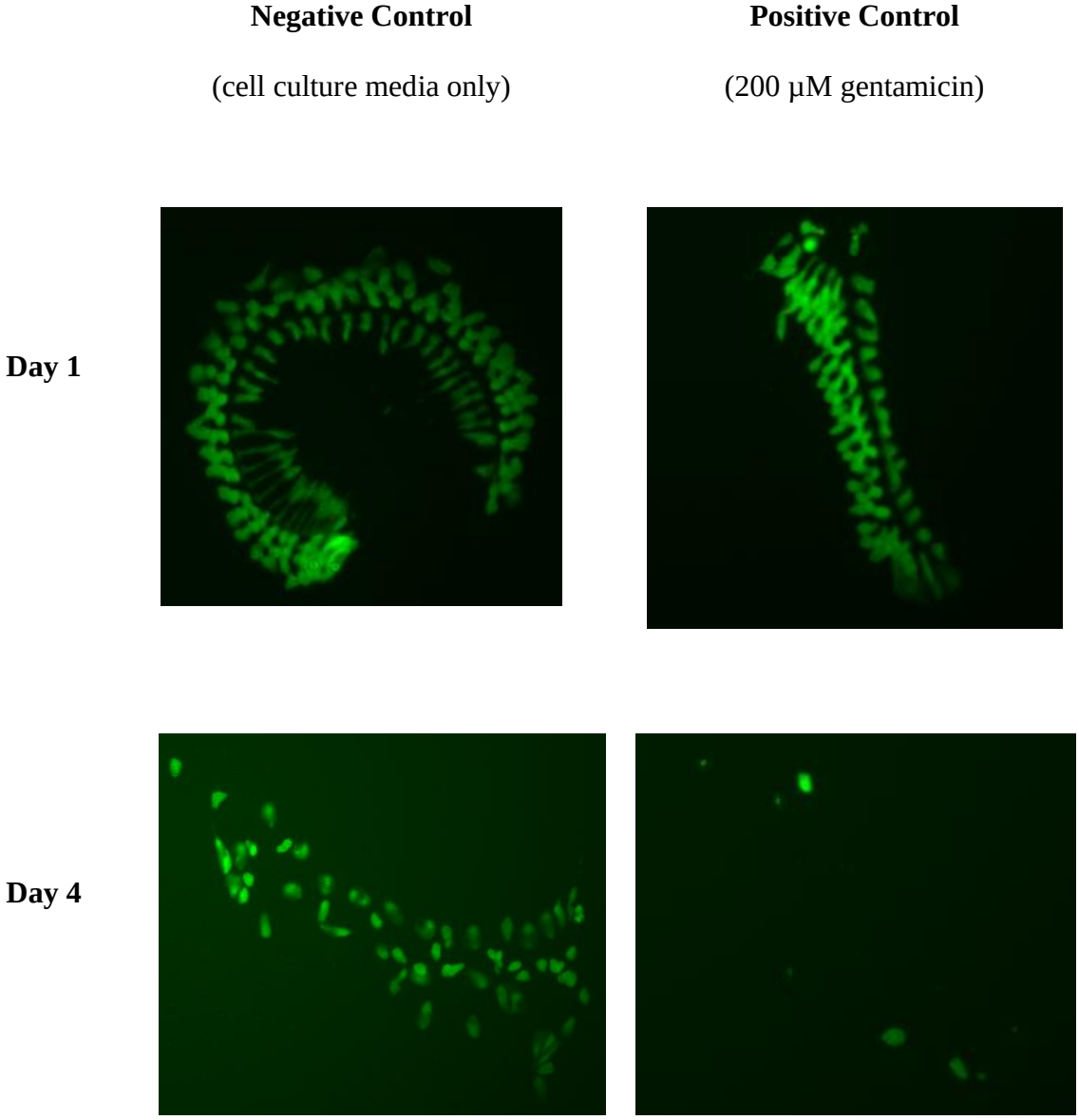

Figure 1: Microexplants from the sensory epithelium of the murine cochlea of *pou4f3*/GFP transgenic mice before treatment with gentamicin (Day 1) and after 3 days of gentamicin exposure (Day 4). The left column shows negative controls where microexplants were treated with only cell culture media, while the right shows positive controls in which HCs were treated with cell culture media + 200 µM gentamicin.

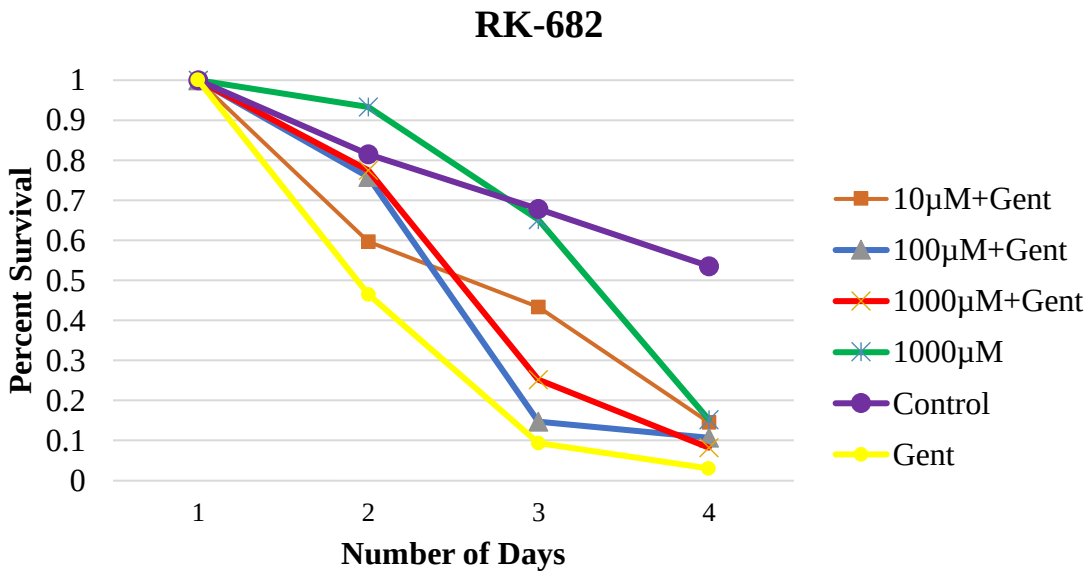

Figure 2: RK-682 showed significant protective ($p>0.05$) at all three experimental doses with gentamicin treatment and showed no significant effects on HC survival at the 1000 µM dose treatment with cell culture media alone.

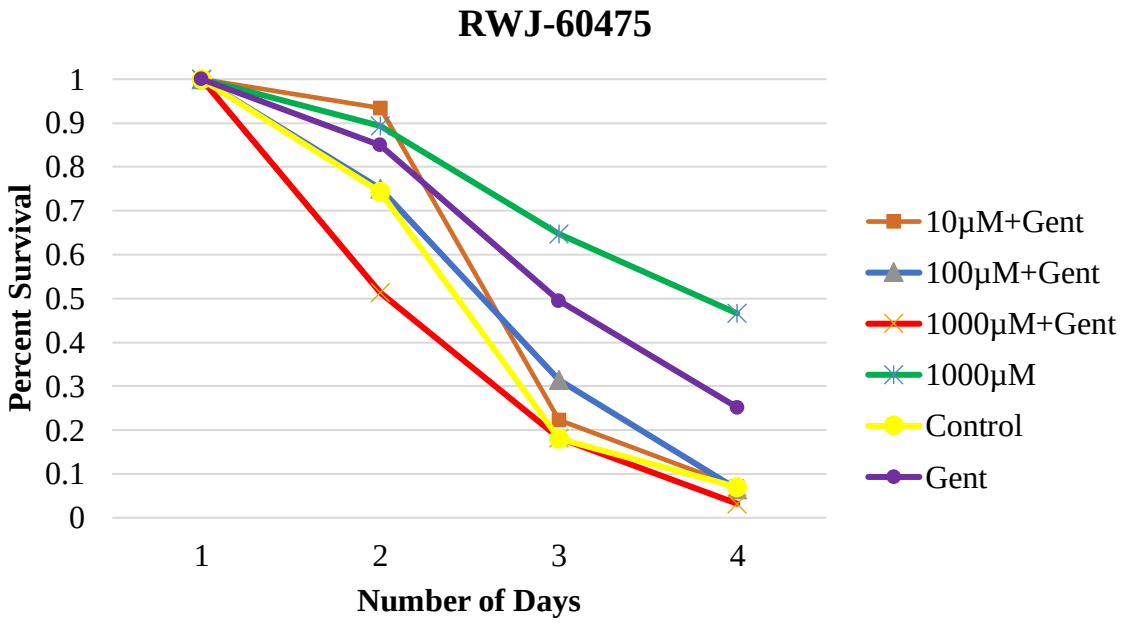

Figure 3: RWJ-60475 showed protective effects at the 10 µM ($p>0.05$) and 100 µM ($p>0.05$) doses with gentamicin treatment and toxic effects at 1000 µM dose with gentamicin treatment. The 1000 µM dose of the compound alone showed no significant effect on HC survival

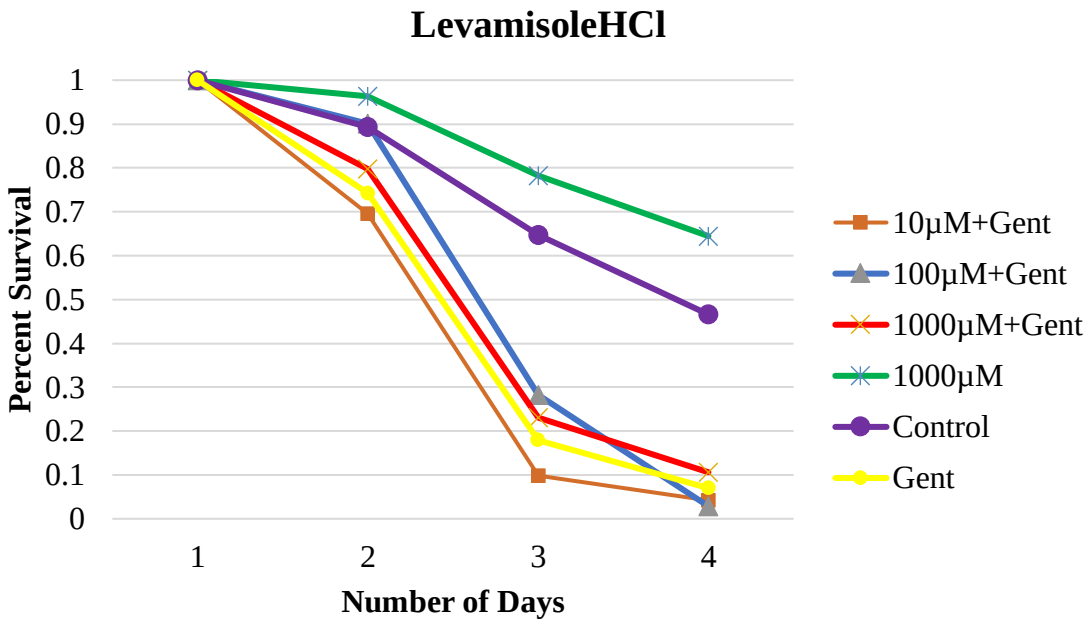

Figure 4: LevamisoleHCl showed significantly protective effects at the 100 µM ($p>0.05$) and 1000 µM ($p>0.05$) doses with gentamicin treatment, while the 10 µM ($p>0.05$) treatment showed toxic effects in the presence of gentamicin. The 1000 µM dose of compound alone showed no significant effect on HC survival.

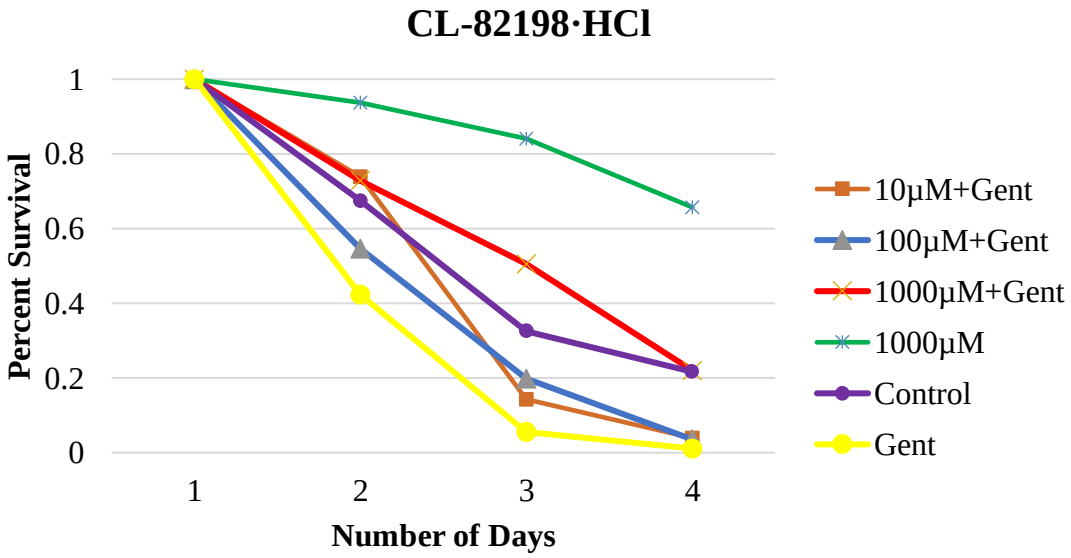

Figure 5: CL-82198HCl showed protective effects at all three experimental doses with gentamicin treatment ($p>0.05$). The inhibitor also showed no significant effects on HC survival or death at the 1000 µM dose alone.

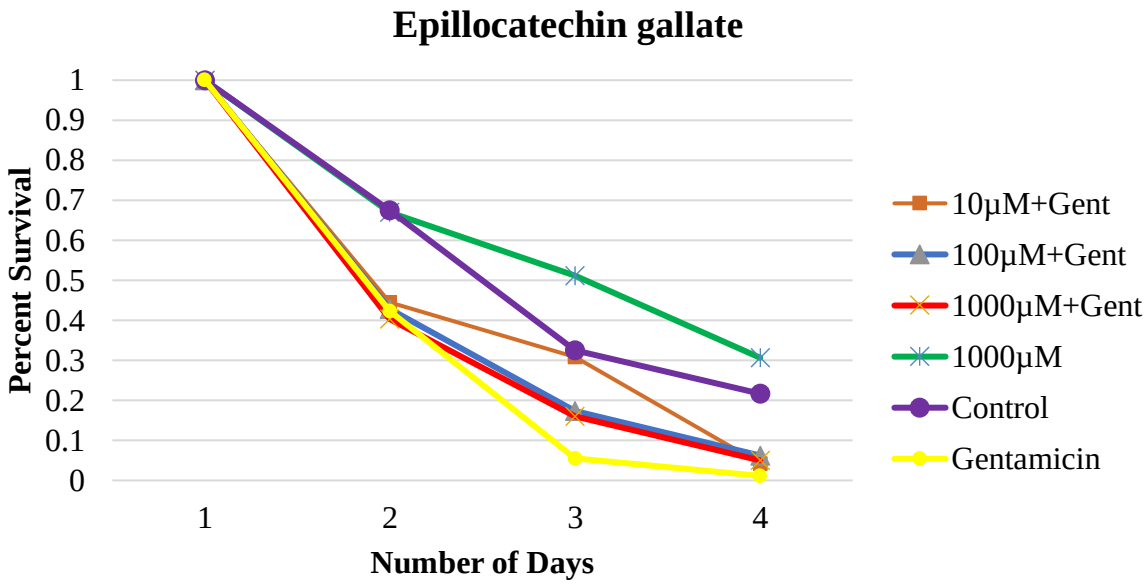

Figure 6: Epillocatechin gallate showed protective effects at all three experimental doses in the presence of gentamicin and the compound alone at the 1000 µM dose had no effect on HC survival.

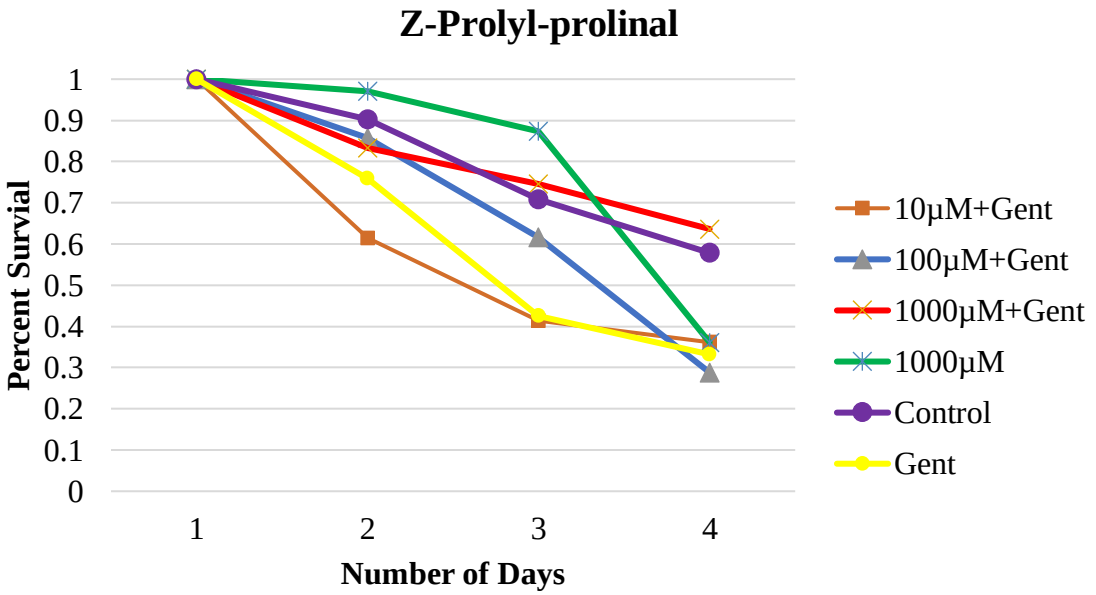

Figure 7: Z-Prolyl-prolinal presented protective effects at the 100 µM ($p>0.05$) and 1000 µM ($p>0.05$) experimental doses with gentamicin treatment. It showed no significant effect on HC survival or degradation at the 1000 µM alone.

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