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PRINCIPAL INVESTIGATOR: Sonia M. Rocha-Sanchez

CONTRACTING ORGANIZATION: Creighton University, Omaha, NE

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Sonia M. Rocha-Sanchez

E-Mail: ssanchez@creighton.edu

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Creighton University
2500 California Plaza
Omaha, NE 68178

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14. ABSTRACT

Post-mitotic mammalian hair cells (HCs) do not regenerate after traumatic damage, and their death leads to irreversible hearing and balance impairment. Therapeutically induced generation of new sensory HCs has been considered for many years. Yet, there are no available pharmacologic alternatives to trigger HC regeneration safely. Our laboratory uncovered the proliferative potential of quinoxaline (Qx), a non-steroidal anti-inflammatory compound, to regenerate lost HCs. During the past year, we focused on two series of Qx analogs to expand the structure of activity relationship of this novel chemotype, identify other proliferative analogs, and optimize potency, bioavailability, and in vivo efficacy. Two lead variants (Qx-294 and Qx-301) have been identified, showing promising *in vitro* ADMETox profiles. Both compounds have been shown to have rapid absorption in zebrafish and induce supporting cell (SC) proliferation in vitro (HEI-OC1 cells, cochleae explants) and in vivo (zebrafish neuromast and mouse inner ear) without signs of apoptosis. Overall, the results support the potential of these two compounds in cochlear HC regeneration.

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NONE LISTED

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TABLE OF CONTENTS

1. INTRODUCTION

Over the past year, we have completed Specific Aims 1 and 2. The outcome of *in vitro* and *in vivo* testing of the two new Qx analogs developed during the present award is described in the next several pages.

2. KEYWORDS

Mammalian auditory hair cells, supporting cells, hearing loss, cell proliferation, cell cycle, hair cell regeneration, medicinal chemistry, drug optimization, quinoxaline, benzopyrazine, NF-κB.

3. ACCOMPLISHMENTS

3.1 Major goals of the project

The following are the primary goals of the project:

Specific Aim 1: To improve Qx efficacy in promoting SC proliferation and differentiation into new HCs through medicinal chemistry, structure-activity relationship (SAR), and *in vivo* pharmacokinetics (PK), and pharmacodynamics (PD).	**Projected Completion Time (in months)**	**Percentage of Completion**
Major Task 1: To modify Qx structure and generate new analogs with improved therapeutic potential		
Subtask 1: To obtain Qx analogs with the following pharmacologic characteristics: 1. IC_{50} lower than the original Qx's IC_{50} (ideally in the nM range). 2. Water solubility at pH 7.4 and stability in solution with $t_{1/2}$ >4 hours. And 3. Show a balance between IC_{50}, potency, efficacy, and ADMET parameters (i.e., absorption, distribution, metabolism, excretion, and toxicity). Optimal Qx analogs will be obtained by the SAR catalog (employing structure databases: ChEMBL, GOSTAR, SciFinder, etc.) and analog synthesis by conventional medicinal chemistry.	1-12	100
Subtask 2: *In vivo* testing of Qx's top analogs. A pilot study carried out using zebrafish to determine the concentration range of the analogs	7-24	100
Subtask 3: To test Qx top analogs' potency (IC_{50}) and toxicity (LD_{50}) in mouse cochlear explants.	7-24	100
Subtask 4: *In vivo* Pharmacodynamics (PD) and Pharmacokinetics (PK) assessments	24-36	100
Milestone(s) Achieved: Generation and identification of new Qx analogs that meet the pharmacologic characteristics defined above with effective proliferative effects in vitro and in vivo.	24	100
Specific Aim 2: Optimize a Qx's oral delivery method to stimulate HC regeneration and characterize its PK/PD properties in the mouse cochleae.		
Major Task 2: To identify the best oral dosage for Qx, which will result in quantifiable inner ear distribution, lead to safer and controlled cell proliferation, and support the design of preclinical and clinical testing		
Subtask 1: Randomized PD assessment of multiple concentration, single oral dose Qx treatment in normal-hearing mice	24-36	100
Subtask 2: Randomized PK assessment of best oral dose determined on subtask 1, at 0.5, 1, 2, 8- and 24-hours post-administration	24-36	100
Milestone(s) Achieved: Identification of optimal oral Qx concentration, which will lead to a consistent and quantifiable proliferative response	36	100

Specific Aim 1: To improve Qx efficacy in promoting SC proliferation and differentiation into new HCs through medicinal chemistry, structure-activity relationship (SAR), *in vivo* pharmacokinetics (PK), and pharmacodynamics (PD)

Summary of Findings

The continued research efforts into the treatment and prevention of hearing loss, such as the promotion of auditory hair cell (HC) regeneration, are still very much needed, even in the wake of all the progress in the auditory field, to date, no pharmacologic alternatives are available to stimulate HC regeneration safely. Studies by Sanchez's laboratory uncovered the proliferative and therapeutic potential of the heterocyclic compound quinoxaline (Qx), a non-steroidal anti-inflammatory compound, to regenerate lost sensorineural HCs. As described in previous progress reports, through medicinal chemistry optimization, we generated a series of quinoxaline derivatives by adding various R groups ranging from hydrophilic to lipophilic. Final compounds were isolated as solids in moderate yields, usually ranging from 24% to 90%. All prepared compounds passed PAINS and Aggregators screening using ZINC15 utility (http://zinc15.docking.org/patterns/home; accessed on Jan 15, 2021). Within the 70 different analogs generated, seven chemotypes: Qx-11, Qx-27, Qx-28, Qx-29, Qx30, Qx-35, and Qx-36 showed potential for further improvements. To further develop and improve the structure-activity relationships of the seven Qx chemotypes, zebrafish larvae expressing HC membrane-bound GFP (*Tg(brn3c: GFP)* were incubated with the top seven Qx-derivatives. A pilot study of 5 larvae per concentration (300 µM and 1 µM) of the different Qx analogs helped us determine the range of working dilutions for each candidate and their toxicologic and solubility properties. Qx-27 provided the best proliferative response at the lowest dose tested with no quantifiable toxicity. Next, we focused on designing an alkyl Qx-27 series (Figure 1).

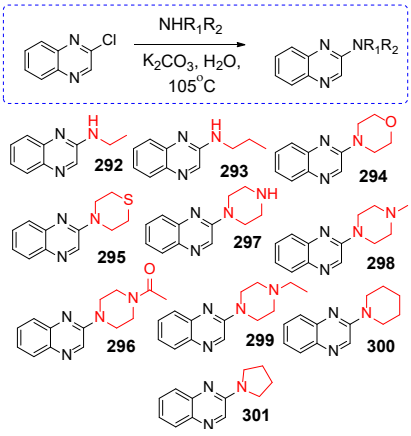


Figure 1: *Synthesis of alkylated Qx27 analogs.* Briefly, 2-chlorquinoxoline was converted to an alkylated Qx-27 analog using 2- chloroquinoxaline, various amines, and potassium carbonate in water heated to 105°C in a sealed pressurized flask overnight. After cooling to room temperature, the precipitate was filtered, washed with cold deionized water, and dried over desiccant overnight. Final products were isolated using normal-phase flash column chromatography. Ten alkylated Qx-27 analogs (Qx-292 – Qx-301) were synthesized and isolated. All compounds, except for Qx-297, 298, and 299, were purified using ethyl acetate in hexane gradient. Compounds 297, 298, and 299 were purified using methanol.

Two of the ten alkylated Qx-27 analogs (Qx-294 and Qx-301) displayed the selection properties that met those sought after in our study: IC_{50} = 15 uM, which is lower than the original Qx's IC_{50} of 25 uM; Water solubility at pH 7.4; stability in solution with $t_{1/2}$ >4 hours; and showing a balance between IC_{50}, potency, efficacy, and ADMET parameters. Both compounds were rapidly absorbed, distributed, and metabolized *in vitro* without significant toxicity (Table 1).

Table 1. Structural variations and cytotoxicity of Qx-294 and Qx-301

Compound Code	Solubility[a] (µg/mL)	Permeability[b] (x10^{-6}cm/s^{-1})	PPB[c] (% Bound)	Metabolic Stability[d] $t_{1/2}$ = hr	Cytotoxicity[e] (IC_{50}, µg/mL)*
Qx-294	71.3±0.3	2.33±0.01	94.1±0.02	5.3±0.2	>20
Qx-301	69.5±2.4	5.61±0.04	94.9±0.3	0.7±0.0	>20

[a]Kinetic solubility assay; [b]PAMPA permeability assay; [c]Mice plasma protein binding; [d]Metabolic stability against human S9 fraction; [e]Cytoxicity was determined against HEI-OC1 cells. *The criteria of cytotoxicity, as established by the National Cancer Institute (NCI) is an IC_{50} < 20 ug/mL

Subsequent studies in zebrafish supported the results obtained from the ADMET assay, showing no quantifiable impact of Qx-294 or 301 treatment on fish swimming behaviors, neuromast morphology,

HC survival, or HC mechanotransduction (data presented in previous progress report). However, consistent with the two compounds' effect on cell proliferation, zebrafish treated with either drug showed clear BrdU incorporation, particularly in the neuromast SCs (Figure 2). Quantification of the BrdU-positive cells revealed a significantly higher number of proliferating cells in animals treated with either compound (‘Figure 2K). However, when compared, neuromasts treated with Qx-301 exhibited more BrdU-positive cells than those treated with Qx-294 (Figure 2K).

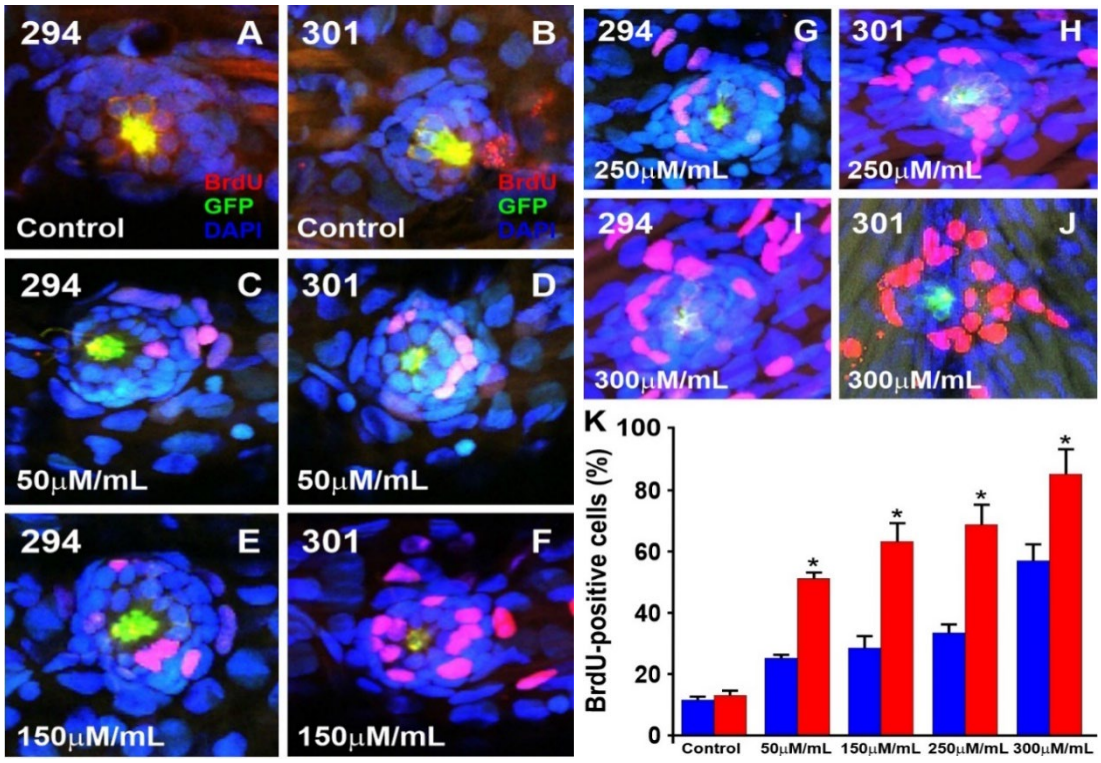


Figure 2: Proliferation assay following Qx-294 or Qx-301 treatment. A, B. No BrdU-labelling (red) was seen in the neuromast’s cells of control animals. C-H. BrdU-positive cells were seen in the SCs surrounding HCs (GFP/green). H. The percentage of BrdU-positive supporting cells per neuromast was calculated for each treatment and represented as mean +/− SEM. One-way ANOVA, *p < 0.05.

To further investigate the effects of Qx-294 and Qx-301 in cochlear HCs, treated explants were fixed and assessed for potential proliferation (BrdU) and cell death (TUNEL). No TUNEL-positive cells were observed in explants treated with either drug (data not shown). Likewise, no BrdU-positive cells were detected. Nevertheless, the presence of several supernumerary HCs in Qx-301-treated explants, particularly in the apical turn, but not in Qx-294- or control (vehicle-only)-treated samples suggests that proliferation happened at some point during the 24-hour incubation period (Figure 3A-C) and at a much higher rate than in the control group (Figure 3D).

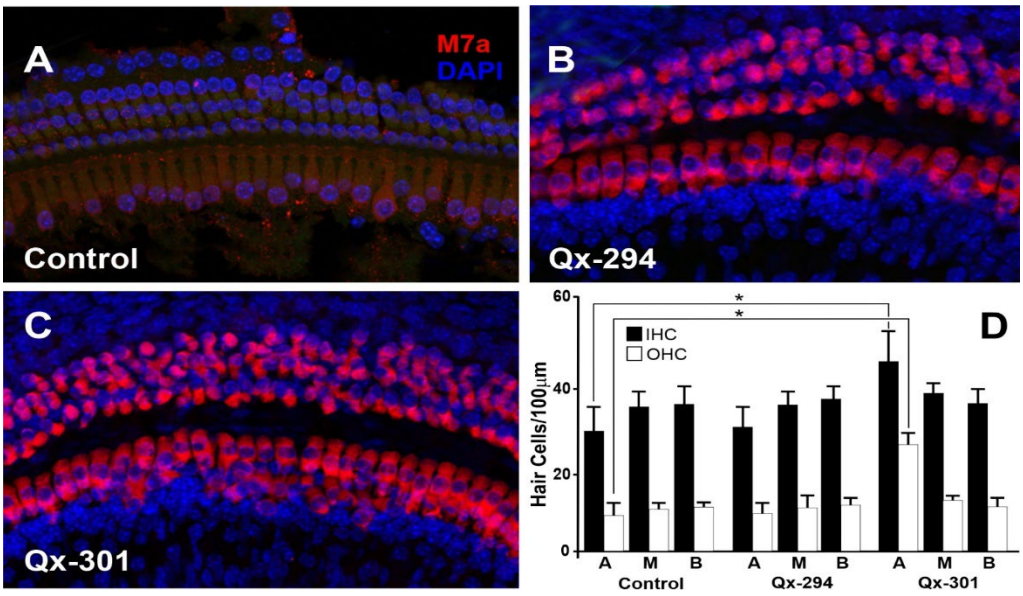


Figure 3: The effect of the two new compounds in cochlear explants. **A-C.** Confocal images of the middle turn in cochlear explant cultures containing **(A)** 0.1% DMSO (control), **(B)** 300 mg/Kg Qx-294, and **(C)** 300 mg/Kg Qx301 for 24 hours. No cytotoxicity to the cochlear explants was observed at the concentration tested. The HC numbers in the three different turns of the cochlear explants are shown

in **(D)**. When treating the cochlear explants with Qx-301 for 24 h, the number of outer hair cells (OHCs) increased from the apical to the basal turn. On the other hand, the number of inner hair cells (IHCs) increased in the apical turn; however, it showed no significant changes in the middle and basal turns. There were no obvious changes in the morphology or arrangement of the OHCs. Nevertheless, supernumerary IHCs were mostly located outside the normal IHC axis in the inner phalangeal and inner border cells' regions. Four cochleae were used for each group. Data are expressed as the mean ± S.E. *p<.05 vs. the control group by one-way ANOVA. ■ = OHC; □ = IHC.

Key Outcomes: Overall, completion of Specific Aim 1 supports the premise that:

1. Qx-294 and Qx-301 have a lower IC_{50} (IC_{50} = 15 uM) than the original compound (IC_{50} = 25 uM) developed in the earlier phases of this study. Both compounds can be rapidly absorbed, distributed, and metabolized *in vitro* without significant toxicity.

2. Qx-294 and Qx-301 *in vivo* treatment leads to the proliferation of mantel cells and the generation of extra HCs in zebrafish neuromast and cochlear explants without any signs of cell death.

3. Treatment with Qx-301 leads to a higher proliferation rate in HEI-OC1 cells, zebrafish neuromast, and cochlear explants than that observed after treatment with Qx-294.

4. Supernumerary cells in the cochlear explants, following Qx-294 and 301 treatment, are likely the result of Deiters' (Outer HCs region) and inner border/inner phalangeal cells (Inner HCs regions) re-entering the cell cycle and proliferating.

5. Neither Qx-294 nor Qx-301 affects HC mechanotransduction, as confirmed by assays with FM1-43 in zebrafish.

Specific Aim 2: Optimize a Qx's oral delivery method to stimulate HC regeneration and characterize its PK/PD properties in the mouse cochleae.

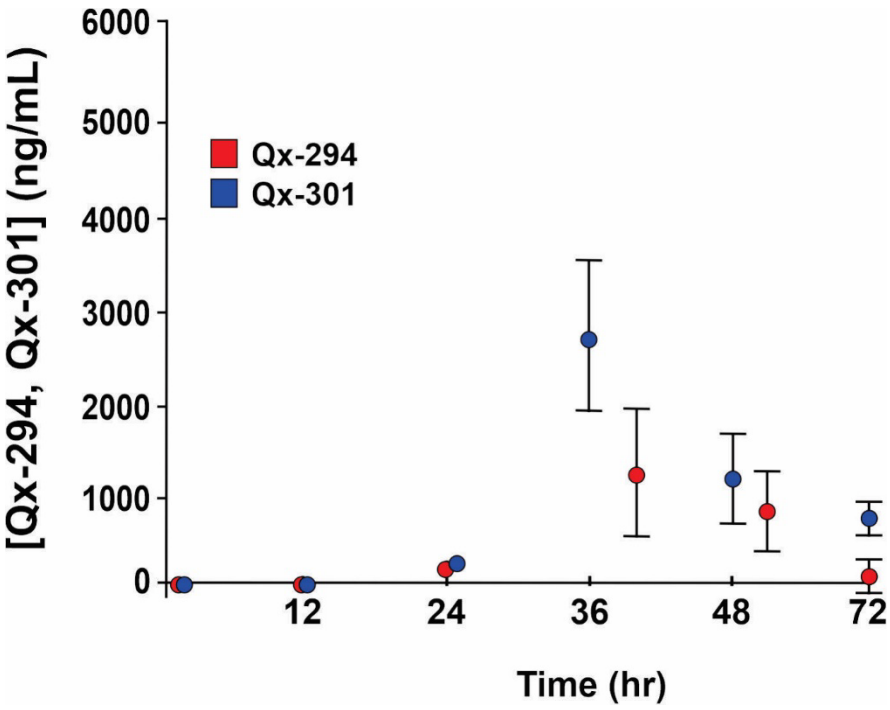


Figure 4. Qx-294 and Qx-301 cochlea distribution following gavage.

Summary of Findings The effective and safe treatment of auditory disorders has become increasingly dependent on inner ear drug delivery systems. For several inner ear disorders (e.g., idiopathic sensorineural hearing loss, Meniere's disease, and noise-induced hearing loss), the oral route of administration is considered the first-line approach. Although convenient, thus far, this route is largely ineffective due to the poor penetration of the blood labyrinth barrier (BLB). Many drugs' clinical usefulness is limited by their systemic toxicity and the adverse effects related to the high doses required to achieve sufficient therapeutic effects (Singh & Kumar Irugu, 2019). On the other hand, the transtympanic route of administration can bypass the BLB and directly access the inner ear through the round window membrane, increase the drug concentration at the targeted organ, and minimize any

potential systemic adverse effects. However, this route requires repeated injections to achieve a prolonged residence in the middle ear and a therapeutic concentration in the inner ear (Lukashkin et al., 2020). To optimize Qx's *in vivo* effect on cell proliferation, we conducted a comparative pharmacokinetic (PK) study on both compounds to determine the effective dose for *in vivo* efficacy trials. A single dose of 300 mg/Kg was administered orally (P.O.) to adult (postnatal day 24 at the time of drug treatment) male and female C57BL6 mice [N=45; three animals per treatment (compounds or control) over five post-treatment time points]. Compound quantification from blood plasma (data not shown) and cochlea tissue (Figure 4) occurred at the 1, 12, 24, 48, and 72-hour time points. No sign of Qx Qx-294 or 301 was observed in the cochlea at any time shorter than 24 hours when traces of the compounds were first detected (Figure 4). No significant differences between compound concentrations were observed in the cochlea (P= 0.053) at any time. However, QX-301 levels were slightly higher than QX-294 at all time points where the compounds could be quantified (Figure 4). Both compounds' concentrations peaked at around 36 hours following gavage (Figure 4), suggesting a slower yet steady accumulation in the cochlea; nevertheless, by 72 hours after treatment, both compounds' concentrations decreased, suggesting a steady elimination and relatively high clearance of both compounds (Figure 4).

Next, we assessed the effect of Qx-294 and Qx-301 treatment in the mouse cochlea anatomy. P24 male and female mice [N = 27; three animals per treatment (compounds or control) over three post-treatment time points] were assigned to different drug treatment groups (Qx-294, Qx-301, vehicle-control) and time of post-treatment evaluation (24, 48, or 72 hours). After each time, animals were perfused and fixed with 4% paraformaldehyde (PFA) overnight at 4 °C. Immunohistochemistry experiments were performed as previously described (Rocha-Sanchez et al., 2018) using Myosin 7a antibody and DAPI counterstaining (Figure 5A-I). The overall organization of the organ of Corti and the number of HCs were assessed (Figure 5J, K). The assessment was performed double-blind and unbiased. One person with no knowledge of the treatment group to which the cochlea belonged performed hair cell counts. Inner and outer HCs were counted on reconstructed confocal stacks using NIH ImageJ v1.8.0 software. The presence of a hair cell was defined as an intact, spherical nucleus located in the basal half of a Myosin 7a-positive cell. The same person who performed the initial counts recounted a select group of cochleae to determine a measure of intra-rater reliability. We used intra-class correlations (ICC) to determine the reliability of our data sets. Reliability statistics were calculated in SPSS v22.0. We used an intra-class correlation coefficient of ≥ 0.7 to measure strong absolute agreement between the two sets of HC counts being compared. Statistical statistical tests examining differences in HC number between treatment groups are ongoing. T-tests will be used to assess differences in the total numbers of HCs (i.e., inner HCs and outer HCs) across treatment groups for both drugs. Supernumerary Myosin 7a-positive cells were observed in the organ of Corti of mice treated with either drug after 48 and 72 hours of treatment (Figure 5A-I). However, when compared to the control (vehicle-only) animals, no major differences in HCs numbers were observed in animals examined 24 hours after gavage feeding, regardless of the compound (i.e., Qx-294 or Qx-301) (data not shown, but the overall anatomy of the organ of Corti was comparable to that of the control sample shown in Figures 5A, D, and G). To examine the effect of Qx-294 and Qx-301 treatment on HC survival, we performed a terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. To detect potential apoptosis in the mouse organ of Corti, we employed the *in situ* cell death detection kit, TMR red (Roche), according to the manufacturer's instructions. Consistent with previous results in zebrafish, no signs of apoptosis were observed in the organ of Corti of mice treated with Qx-294 or Qx-301 in any treatment group (data not shown). Mechanotransduction channels are at the core of sensory HCs' activity. The styryl pyridinium dye FM1-43 provides an optical measure of ion channel function and, therefore, used as a proxy for the viability of the neuromast cells treated with QX-291 or Qx-301, as well as indirect functional information for the newly formed HCs. FM1-43FX (Thermo Fisher) uptake experiments were performed as previously described (Rocha-Sanchez et al., 2018). Animals were fed with Qx-294, Qx-301 at 300 mg/Kg, or vehicle-only (control) 48 hours before injection with E3. Two days later, animals were injected with 3 μM of FM1-43FX for 10 minutes, euthanized, and the cochleae were dissected for immunohistochemistry with Phalloidin and counterstained with DAPI (Figure 6A-C).

The fluorescence incorporated was quantified according to (Rocha-Sanchez et al. (2018) using ImageJ. Similar to our previous findings in zebrafish, quantification of the fluorescence intensity incorporated by the neuromasts did not show any significant differences between treatment or control groups (data not shown), indicating that neither Qx-294 nor Qx-301 block the mechanotransduction channels and supporting the premise that Qx-induced, newly generated HCs in the mouse organ of Corti are functional.

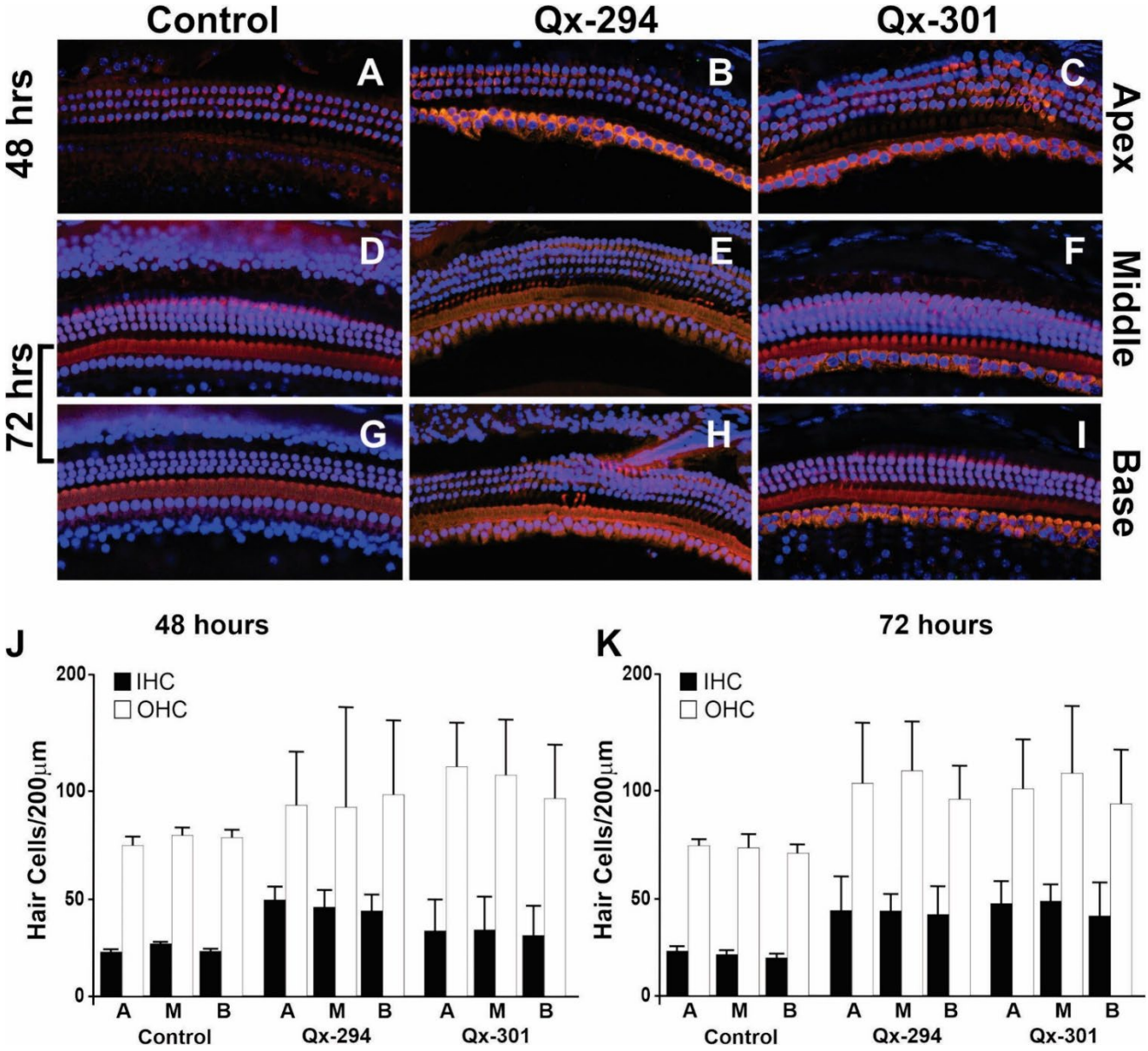


Figure 5. Oral administration of Qx-294 and Qx-301 does not affect the integrity or survival of the organ of Corti HCs *in vivo*. Compared to control (vehicle-only) animals **(A, D, G)**, mice treated with either Qx-294 **(B, E, H)** or Qx-301 **(C, F, I)** showed no changes to the integrity and anatomy of the sensory epithelia. Notably, supernumerary Myosin 7a-positive cells were observed at the inner and outer HC regions of compound-treated **(B-I)** but not control animals **(A, D, G)**, particularly in the organ of Corti of mice examined at 48 and 72 hours after drug treatment. Due to the density of those supernumerary cells, we have chosen not to label them with arrows; however, they are promptly visible in the pictures. No changes in cell numbers were observed between the control group and mice examined 24 hours after treatment. **(J, K)**. Quantification of the total numbers of outer and inner HCs revealed a marked increase in sensory HC numbers in compound-treated animals. We are currently evaluating those results statistically. The number of supernumerary cells per samples was calculated for each treatment and represented as mean +/− SEM. Red = Myosin 7a; Blue = DAPI.

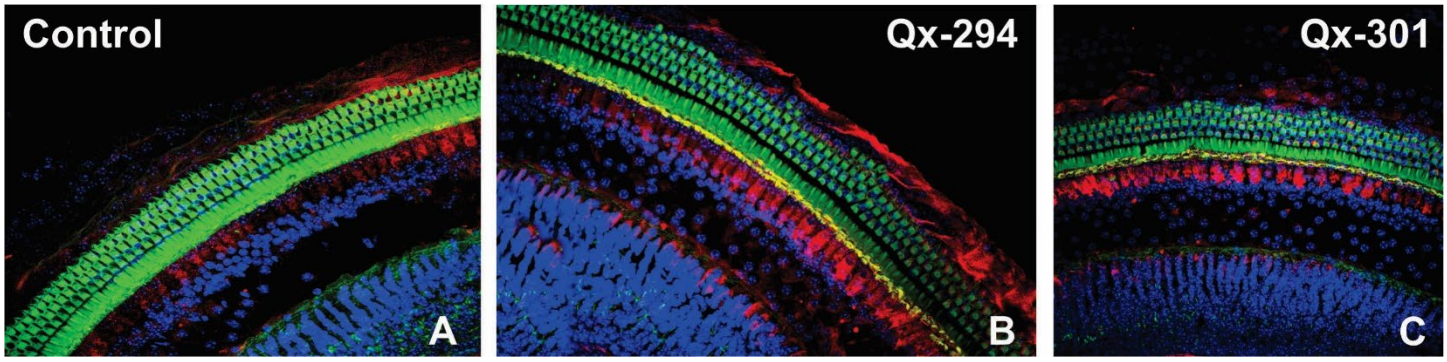
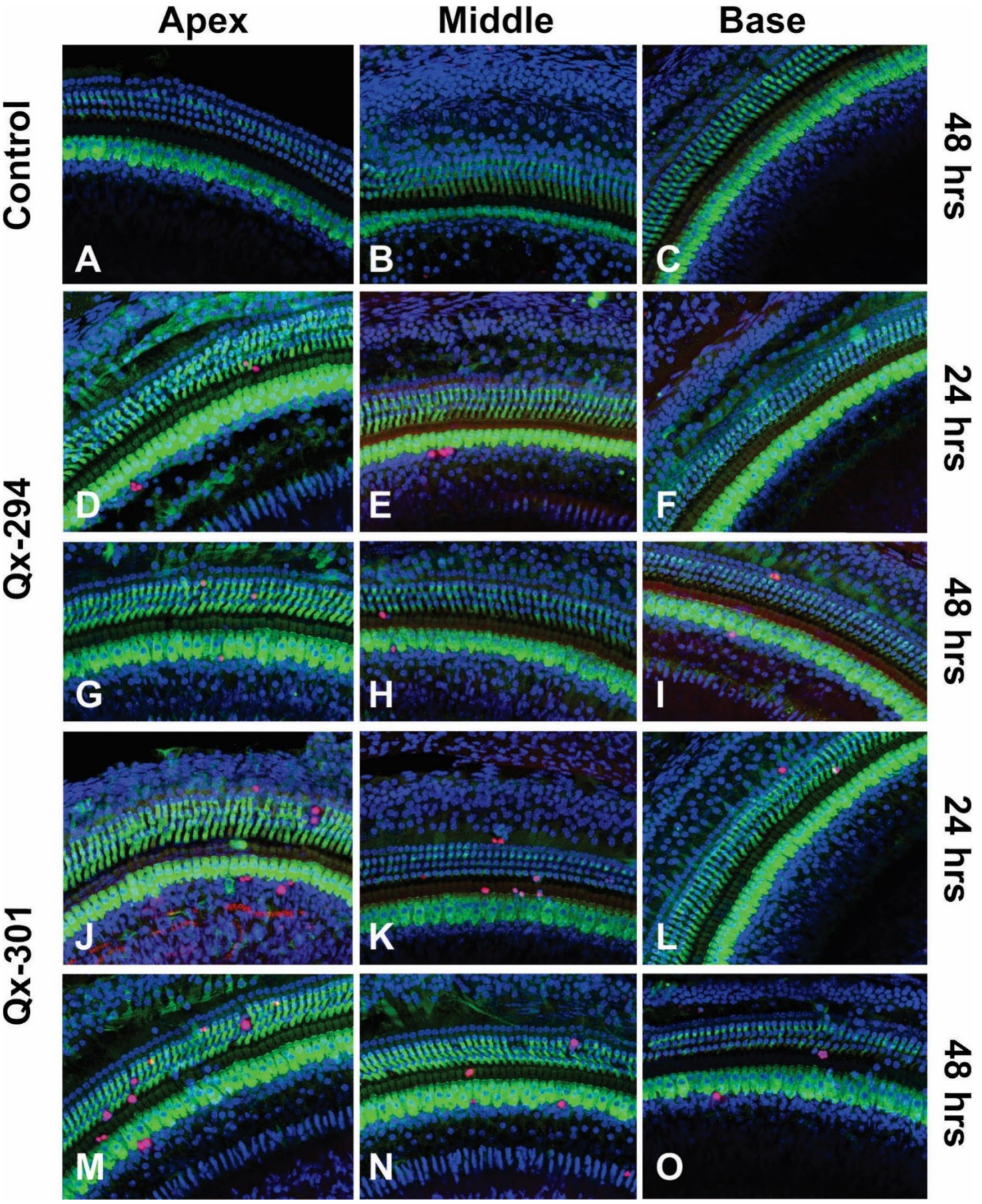


Figure 6. Treatment with Qx-294 or Qx-301 does not abolish mechanotransduction channel activity in the mouse organ of Corti. Consistent with the results presented above (Figure 5), a comparison with the control samples **(A)** revealed the presence of supernumerary cells **(B, C)** in the organ of Corti of compound-treated animals. Of note, only the apical turns for each treatment are shown above. Red = FM1-43; Blue = DAPI; Green = phalloidin.

To further assess how orally administered Qx-294 and Qx-301 affected the cochlea, we evaluated the proliferative potential of both compounds in the mouse organ of Corti (Figure 7A-O). To that end, on the last day of the post-treatment waiting time (i.e., 24, 48, 72 hours), mice were injected with the thymidine analog EdU (50 mg/kg) and killed 4 hours after injection. Three animals were used per time point and compound, and the experiments were repeated three times. All animals were P24 at the beginning of the experiments. EdU incorporation was analyzed by immunohistochemistry. Quantification of EdU-positive cells and analyses of potential differences between compounds and post-treatment waiting time per compound are being analyzed. As such, no data will be presented at this time. Consistent with their potential effect on HC regeneration, EdU-positive cells were observed in the organ of Corti of mice treated with either Qx-294 (Figure 7D-I) or Qx-301 (Figure 7J-O), particularly between the 24 and 48-hour period. Although supernumerary cells were observed in the OC of animals analyzed 72 hours after treatment (data not shown), the lack of EdU-positive cells in those mice suggests that most proliferation may have taken place around 36 hours post-treatment when the concentration of both compounds was found to be at their highest concentration in the cochlea (Figure 4). Those Edu-positive cells were observed mostly around the Deiter's and inner border/inner phalangeal cells' region. No proliferating cells were observed in the organ of Corti of control mice. Functional evaluation of the auditory functions after treatment with each compound [i.e., Auditory brainstem responses (ABR) and distortion product otoacoustic emissions (DPOAE)] were carried out before and after drug treatment. That data is currently under analysis and will be presented in the final Progress Report. We have been approved for an additional six months of non-cost extension to complete all analyses.

Figure 7. Proliferation assay following Qx-294 or Qx-301 at 24 hours, 48 hours, and 72 hours (not shown) treatments. **A-C.** No BrdU-labelling (red) was seen in the organ of Corti of control animals, regardless of the treatment time (data shown only for a 48-hour sample)**. D-O**. BrdU-positive cells were seen in the outer and inner HCs (green) regions at 24 and 48-hour intervals. While supernumerary HCs were observed in samples processed after 72-hours treatment (see Figure 5), no proliferating (BrdU-positive) cells were observed at that same time point, suggesting that proliferation may have happened earlier between 24-48 hours post-treatment. Red = BrdU; Blue = DAPI; Green = Myosin 7a.


Apex
Middle
Base
Control
Qx-294
Qx-301
48 hrs
24 hrs
48 hrs
24 hrs
48 hrs
A
B
C
D
E
F
G
H
I
J
K
L
M
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O

Key Outcomes: Overall, completion of Specific Aim 2 supports the premise that:

1. Orally administered Qx-294 and Qx-301 can pass the blood-labyrinth barrier.

2. Oral administration of Qx-294 and Qx-301 results in a quantifiable proliferative response yet more contained than what we have previously observed with injectable treatment.

3. Some newly proliferated cells differentiate into HC-like cells, as presumed from the presence of Myosin 7a-positive supernumerary cells in the treatment groups but not in the controls.

4. Supernumerary cells in the cochlear explants, following Qx-294 and 301 treatment, are likely the result of Deiters' (Outer HCs region) and inner border/inner phalangeal cells (Inner HCs regions) re-entering the cell cycle and proliferating.

5. Neither Qx-294 nor Qx-301 affects HC mechanotransduction, as confirmed by assays with FM1-43 in zebrafish.

References

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Rocha-Sanchez SM, Fuson O, Tarang S, Goodman L, Pyakurel U, Liu H, He DZ, Zallocchi M. Quinoxaline protects zebrafish lateral line hair cells from cisplatin and aminoglycosides damage. Sci Rep. 2018 Oct 11;8(1):15119. doi: 10.1038/s41598-018-33520-w. Erratum in: Sci Rep. 2019 Jun 14;9(1):8720. PMID: 30310154; PMCID: PMC6181994.

Singh A, Kumar Irugu DV. Sudden sensorineural hearing loss - A contemporary review of management issues. .J Otol. 2020 Jun;15(2):67-73. doi: 10.1016/j.joto.2019.07.001. PMID: 32440269.

3.3 Opportunities for training

Nothing to report

3.4 Disseminating the results to the community of interest

The PI presented partial results of the present study at the "16th Winter Conference in Medicinal & Bioorganic Chemistry, which took place in February of 2023 in Steamboat, CO.

3.5 Plans for the next reporting period

The next reporting period will consist of the final results from the present study. We plan to have a manuscript submitted for publication by then.

1. IMPACT

4.1 Impact on the development of the principal discipline(s) of the project

Nothing to report

4.2 Impact on other disciplines

Nothing to report

4.3 Impact on technology transfer

Nothing to report

4.4 Impact on Society beyond science and technology

Nothing to report

2. CHANGES/PROBLEMS

5.1 Changes in approach and reasons for change

No changes have been made to our original research plan.

5.2 Actual or anticipated problems or delays and actions or plans to resolve

Nothing to report

5.3 Changes that had a significant impact on expenditures

No changes to report

5.4 Significant changes in use or care of human subjects, vertebrates, animals, biohazards, and/or select agents

No changes to report

3. PRODUCTS

6.1 Publications, conference papers, and presentations

6.1.1 Journal publications

Bhattarai P, Hegde P, Li W, Prathipati P, Stevens C, Yang L, Zhou H, Pandya A, Cunningham K, Grissom J, Roman-Sotelo M, Sowards M, **Calisto L**, Destache C, **Rocha-Sanchez SM**; Gumbart J, Zgurskaya H, Jackson M, North, EJ. Structural Determinants of Indole-2-carboxamides: Identification of Lead Acetamides with Pan Antimycobacterial Activity. *Journal of Medicinal Chemistry*. *J. Med. Chem.* 2023, 66, 1, 170–187.

6.1.2 Books or other non-periodical, one-time publications

Nothing to report

6.1.3 Other publications, conference papers, and presentations

- Grissom J, Calisto LE, **Rocha-Sanchez SM**, North EJ. Structural Determinants of the Highly Potent Indole-2 Carboxamide Antimycobacterial Agents. *16th Winter Conference on Medicinal & Bioorganic Chemistry*. Steamboat, CO. January 2023.

- Calisto LE, Menghini A, Barthol B, Cunningham KM, North EJ, **Rocha-Sanchez SM.** Pharmacologic Regulation of Auditory Sensory Hair Cell Regeneration. *16th Winter Conference on Medicinal & Bioorganic Chemistry*. Steamboat, CO. January 2023.

- Cunningham K., Calisto L., **Rocha-Sanchez SM**, North EJ. Design, Synthesis and Biological Evaluation of Novel Antimycobacterial Acetamides. *16th Winter Conference on Medicinal & Bioorganic Chemistry*. Steamboat, CO. January 2023.

6.2 Website(s) or other Internet sites

Nothing to report

6.3 Technologies or techniques

Nothing to report

6.4 Invention, patent applications, and/or licenses

Nothing to report

6.5 Other Products

Nothing to report

4. PARTICIPANTS AND OTHER COLLABORATING ORGANIZATIONS

7.1 Project Personnel

Name:	Sonia Rocha-Sanchez
Project Role:	Principal Investigator
Research Identifier:	https://orcid.org/0000-0001-5119-3891
Nearest person month worked:	12
Contribution to the project:	Dr. Sanchez has performed work in drug design, testing, and data analyses.
Funding Support:	This award

Name:	Lilian Calisto
Project Role:	Laboratory technician
Research Identifier:	https://www.researchgate.net/profile/Lilian-Calisto
Nearest person month worked:	12
Contribution to the project:	Ms. Calisto performed a variety of general tasks (animal care and handling, lab bench work, ordering, etc.) associated with the study
Funding Support:	This award

Name:	Broch Barthol
Project Role:	Work-Study Student
Research Identifier:	None
Nearest person month worked:	6
Contribution to the project:	Broch assisted with immunohistochemistry, tissue dissection, cell counting, and *in vitro* drug treatments associated with the study
Funding Support:	This award

Name:	Kenneth Nguyen
Project Role:	Work-Study Student
Research Identifier:	None
Nearest person month worked:	6
Contribution to the project:	Ken assisted with immunohistochemistry, tissue dissection, cell counting, and *in vitro* drug treatments associated with the study
Funding Support:	This award

7.2 Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to report

7.3 What other organizations were involved as partners?

Nothing to report

5. SPECIAL REPORTING REQUIREMENTS

8.1 Collaborative Awards

NIH/P20 GM139762-01 (Research Project Mentor: E. Jeffrey North, PI: Peter Steyger), Title: Development of Novel Therapeutics for Treatment of Mycobacterial Infections, 07/12/2021 – 06/30/2024

8.2 Quad Charts

Appendix 1

Appendix

Pharmacologic Regulation of Auditory Hair Cell Regeneration

W81XWH-20-1-0789
RH190008

PI: Sonia M. Rocha-Sanchez **Org:** Creighton University **Award Amount:** $363,750

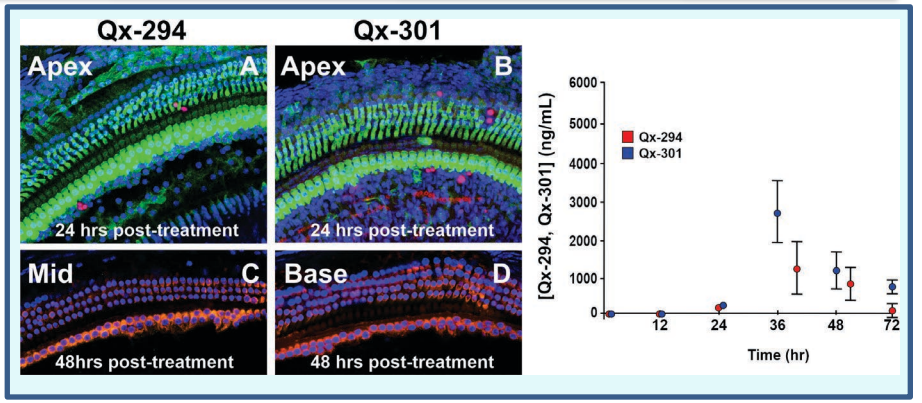
Study/Product Aim(s)

SA1: Improve quinoxaline (Qx) efficacy in promoting SC proliferation and differentiation into new HCs through medicinal chemistry, structure-activity relationship (SAR), and *in vivo* pharmacokinetics (PK) and pharmacodynamics (PD).

SA2: Optimize a Qx's oral delivery method to stimulate HC regeneration and characterize its PK/PD properties in the mouse cochleae.

Approach

As an outcome of this study, two lead variants (Qx-294 and Qx-301) were identified, which showed promising *in vitro* ADMETox profiles. *In vivo* analyses confirmed those variants' potential to elicit cell division in zebrafish neuromast and the mouse organ of Corti. Both compounds have been shown to have rapid absorption in zebrafish and mice (oral gavage) without signs of apoptosis. Overall, the results support continuing the studies in a mammalian (mouse) system to evaluate those compounds' potential to trigger hair cell regeneration after insult to the sensory epithelia.



Qx-294 and Qx-301 have been identified, which show promising *in vitro* ADMETox profiles (see report for data). Both compounds have IC_{50} = 15 **(A)** and stable proliferative effect on neuromast (previous report) and inner ear Supporting cells **(A-D)**. Following gavage, both compounds can be detected in the cochlea, peaking around 36 hours after treatment.

Timeline and Cost

Activities CY	20-21	21	22	23
Modification of Qx original structure				
Generation of new Qx analogs				
In vitro and *in vivo* testing of new analogs				
Identify the best oral dosage for Qx in vivo				
Estimated Budget ($K)	$181,875	$181,875		

Updated: (09/27/2023)

Goals/Milestones

Cy20/21 Goal – To obtain Qx analogs with the following pharmacologic characteristics:

- ☑ IC_{50} lower than original Qx's IC_{50} (ideally in the nM range)
- ☑ Water solubility at pH 7.4, and stability in solution with $t_{1/2}$ >4 hours
- ☑ Show a balance between IC_{50}, potency, efficacy, and ADMET parameters (i.e., absorption, distribution, metabolism, excretion, and toxicity)

Cy21/22 Goals – *In vivo* testing of Qx's top analogs

- ☑ To test Qx top analogs' potency (IC_{50}) and toxicity (LD_{50}) in mouse cochlear explants
- ☑ *In vivo* Pharmacodynamics (PD) and Pharmacokinetics (PK) assessments

Cy22/23 Goal – Production Readiness

- ☑ Randomized PD assessment of multiple concentration, single oral dose Qx treatment in normal-hearing mice
- ☑ Randomized PK assessment of best oral dose determined on subtask 1, at 0.5, 1, 2, 8- and 24-hours post administration

Comments and Challenges: We have completed all experiments and requested a six-month extension to complete data analyses.