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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 the zebrafish neuromast and cochlea explants without signs of apoptosis. Overall, the results support the continuation of the studies in vivo in a mammalian (mouse) system.

15. SUBJECT TERMS
None listed.

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1. INTRODUCTION

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. Preliminary findings underscored the therapeutic potential of the heterocyclic compound quinoxaline (Qx) (a.k.a. benzopyrazine) - a non-steroidal anti-inflammatory drug - and some of its derivatives to stimulate HC regeneration and treat hearing loss. Qx has manifold biological properties, which seem to depend on how it is metabolized within cells (cell-dependent effect), dosage (dose-dependent effect), modifications of its chemical structure, and length of treatment. Based upon that premise, we applied medicinal chemistry to (1) improve Qx efficacy in promoting supporting cell (SC) proliferation and differentiation into new HCs and (2) optimize Qx potency for oral delivery. We have generated several analogs (Qx1 – Qx 70) and tested those variants *in vitro* (HEI-OC1 cells) and *in vivo* (zebrafish) for their efficacy in promoting supporting cell (SC) proliferation. During this 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, optimize potency and bioavailability, and *in vivo* efficacy. Two lead variants (Qx-294 and Qx-301) have been identified, which show promising *in vitro* ADMETox profiles. Both compounds have been determined to show no cytotoxicity against HEI-OC1 cells, are permeable, have high plasma protein binding, and are metabolically stable. When tested *in vivo*, both compounds have been shown to have rapid absorption in zebrafish and induce neuromast SC proliferation without any signs of cytotoxicity. Overall, the results gathered so far support further development of these novel compounds to stimulate HC regeneration and hearing restoration.

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

Over the past year, we have completed most of Specific Aim 1. Moreover, we identified two new Qx analogs that meet the pharmacologic characteristics defined in the original application and display proliferative effects *in vitro* and *in vivo*.

3.1 Major goals of the project

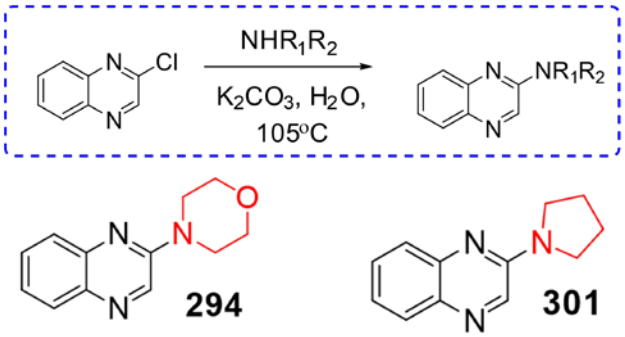
The following are the primary goals of the project:

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	60
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	80
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	10
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	10
Milestone(s) Achieved: Identification of optimal oral Qx concentration, which will lead to a consistent and quantifiable proliferative response	36	20

3.2 Current accomplishments under proposed goals

We have conducted experiments described on Specific Aim 1, major task 1 (subtasks 1-4), completed subtasks 1-3 and completed about 60% of subtask 4. Moreover, we have started experiments towards the completion of Specific Aim 2. Specifically, we have identified two analogs, Qx-294 and Qx-301, which displayed the selection properties (i.e., IC_{50} lower than original Qx's IC_{50}, Water solubility at pH 7.4 and stability in solution with $t_{1/2}$ >4 hours, and showing a balance between IC_{50}, potency, efficacy, and ADMET parameters). Results of the *in vitro* (HEI-OC1 cells and cochlear explants) and *in vivo* (zebrafish) are shown as follows:



Scheme 1. Synthetic Pathway for the production of Qx analogs. Percentage yield Qx-294: 22%; Qx-301: 46%. Analogs were characterized with ^{1}H NMR, ^{13}C NMR, mass spectrometry, and HPLC.

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).

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:

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

Specific Objective: To develop an improved Qx formulation with better pharmacological characteristics (as specified in the table above) than the original Qx formulation.

Key Outcomes: Scheme 1 shows the synthesis of the alkylated Qx27 analogs. Briefly, 2-chlorquinoxoline is converted to an alkylated Qx27 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 is filtered, washed with cold deionized water, and dried over desiccant overnight. Final products were isolated using normal-phase flash column chromatography. Compounds were purified using ethyl acetate in hexane gradient and molecularly characterized using mass spectrometry and proton (^{1}H) nuclear magnetic resonance.

In vitro ADMETox

Cytotoxicity

The cytotoxicity of the synthesized compounds was screened in the HL-60 cell line by adopting the well-established MTT colorimetric microculture assay method (Rocha-Sanchez et al., 2018). Increasing concentrations of each drug were placed with HEI-OC1 cell suspension (5×10^4 cells/well). Cell growth was determined 72 h later by adding 50 µL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (2.5 mg/mL). This was reduced by mitochondrial dehydrogenase of viable cells in an insoluble blue formazan product during the 4 h contact period at 37°C. After the supernatant was removed, the formazan crystals were solubilized by adding DMSO (100 µL). These plates were read at 550 nm with an enzyme-linked immunosorbent assay (ELISA) reader. At each dose level of the compounds tested, cell growth inhibition was expressed as a fractional decrease of 550 nm absorbance in the treated cultures with respect to control and reference compounds (cisplatin and 5-fluorouracil). Results of QX-294 and QX-301 cytotoxicity evaluation against HEI-OC1 cells are summarized in Table 1. Both compounds exhibited no significant toxicity against the HEI-OC1 cell line, reaching an IC_{50} value of 15 uM, after 72 hours of incubation (Figure 1).

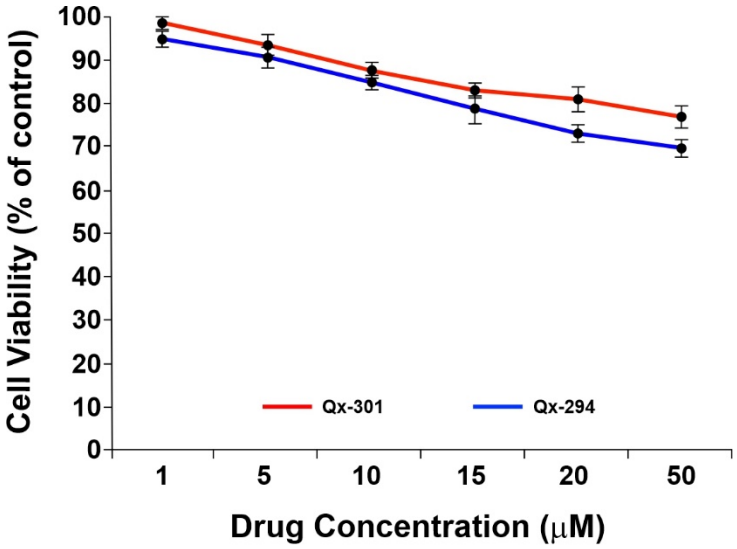


Figure 1: MTT assay to determine the IC50 value of Qx-294 and Qx-301 and analyze their effect on cell viability.

Solubility

Solubility has become a key parameter during drug development, as poor solubility is one of the main causes of poor bioavailability (Avdeef, 2001; Kerns et al., 2008; Lipinski, 2000). For determination of kinetic solubility, 2 µL of a 0.010 mol/L DMSO stock solution was pipetted into one well of a 96 well plate (Millipore MultiscreenHTS-PCF Filter Plate, Art. No. MSSLBPC50) containing 98 µL of phosphate buffer pH 7.4. Following shaking of the suspension on an orbital shaker at 250 rpm for 2 h, it was separated by centrifugation (2500 rpm for 3 min). Immediately after the filtration step, 50 µL of the filtrate was transferred into 50 µL of a 1:1 (v:v) mixture of DMSO (Merck KGaA, Art. No. 1.02950) with phosphate buffer pH 7.4, to avoid precipitation from the saturated solution. The concentration of the research compound was determined by HPLC with UV detection as described below using an external standard from the same batch of research compounds. For the majority of research compounds, crystallinity was not checked. However, to get a better understanding of the behavior of the solid residue

in the kinetic solubility assay, crystallinity was assessed more in-depth for a smaller subset of nine commercial active pharmaceutical ingredients. For these compounds, kinetic solubility was determined as described above, and the assay was stopped after 15 min, 30 min, 60 min, 120 min, 24 h, and 48 h. At the end of the respective assay, concentration in the liquid phase was determined by HPLC, and crystallinity was assessed by polarized light microscopy. A good goal for the solubility of drug discovery compounds is >60ug/mL. Consistent with that, solubility for both Qx-294 and Qx-301 was determined to be higher than 60ug/mL (Table 1).

Permeability

Compound activity at intracellular targets or good absorption after oral dosing requires lipid membrane permeation. Permeability *in vivo* is a complex phenomenon involving several possible mechanisms: passive diffusion, paracellular, active transport, and efflux (Di et al., 2020). Di et al (2020) have estimated that the predominant mechanism of gastrointestinal (GI) absorption for most commercial drugs is passive diffusion. Therefore, compound selection and optimization for passive diffusion is an effective approach. Permeability data can provide an early estimation of barriers to GI absorption, cell assay membrane permeation, and bioavailability. Permeability through specialized *in vivo* membranes, such as the blood-brain barrier (BBB), can be predicted using specialized cell culture models (Di et al., 2003). The parallel artificial membrane permeability assay (PAMPA), first introduced by Kansy, et al. (2004), has been widely used in the pharmaceutical industry as a high throughput permeability assay to predict oral absorption. For permeability assessment, we followed a modified PAMPA assay (Di et al., 2003) using porcine brain lipids to improve the prediction of BBB penetration. Quality control standards, Verapamil (P_e = 16 x 10^{-6} cm s^{-1}) for high permeability and theophylline (P_e = 0.12 x 10-6 cm s^{-1}) for low permeability, were ran with each sample set to monitor the consistency of the analysis. Following the patterns established in the literature for BBB permeation prediction, Qx-294 displayed an uncertain BBB permeation (>2 x10^{-6} cm/s^{-1} and < 4 x10^{-6} cm/s^{-1}), while Qx-301 showed a high BBB permeation (> 4 x10^{-6} cm/s^{-1}) (Table 1).

Plasma Protein Binding (PPB)

Protein binding may affect drug activity in one of two ways: either by changing the effective concentration of the drug at its site of action or by changing the rate at which the drug is eliminated, thus affecting the length of time for which effective concentrations are maintained. Generally, agents that are minimally protein-bound penetrate tissue better than those that are highly bound, but they are excreted much faster. Among drugs that are less than 80-85 percent protein bound, differences appear to be of slight clinical importance. Agents that are highly protein bound may, however, differ markedly from those that are minimally bound in terms of tissue penetration and half-life. The determination of the free fraction (f_u) of a drug via distribution between erythrocytes and plasma was performed as previously described (Schuhmacherer al., 2000). Briefly, erythrocytes were obtained by the centrifugation of fresh, heparinized mice blood, which was then washed three times in isotonic potassium phosphate buffer, pH 7.4. Isolated erythrocytes were resuspended in phosphate buffer, in 10x-diluted plasma, or in undiluted plasma to yield a hematocrit (Hk) of 0.4. Each compound (Qx-294 and Qx-301) was added to erythrocyte suspension to obtain a final concentration of 30 ng/mL. Suspensions were incubated at room temperature for 30 min using a laboratory shaker. After incubation, aliquots of 100mL for determining the total concentration in the erythrocyte suspension were collected. Then, erythrocytes were separated by centrifugation at 1800*g* for 10 min, and 100mL aliquots were aspirated from the resulting supernatants to determine the concentration in buffer or plasma supernatants. Concentrations in the erythrocyte suspension, buffer, and plasma supernatants were determined by LC-MS/MS. Using the unbound fraction (f_u) method, the f_u of Qx-294 and Qx-301 in mouse plasma was found to be 0.6 and 0.4%, respectively. On the other hand, Qx-294 and Qx-301 fractions bound to plasma proteins were 94.1 and 94.9%, respectively (Table 1).

Metabolic Stability

Metabolic stability refers to the susceptibility of compounds to biotransformation in the context of selecting and/or designing drugs with favorable pharmacokinetic properties. Liver S9 fractions (the 9000g supernatant of a liver homogenate) are not only easily obtained during the early stages of liver microsomal preparation (Li 2001; Moghaddam 2008), but they also contain both microsomal and cytosolic fractions that can provide more metabolic information than microsomes alone. This is because microsomes lack cytosolic enzymes. Homogenization and differential centrifugation of liver tissue enables the concentrated source of enzymes available in the S9 fraction. Unlike liver microsomes, which contain only the endoplasmic reticulum subcellular fraction (containing most notably cytochrome P450's or CYPs and Uridine 5'-diphospho-glucuronosyltransferase; UGTs), the S9 fractions further contain the cytosolic enzymes such as aldehyde oxidase, xanthine oxidase, sulfotransferases, methyltransferases, N-acetyl transferases, and glutathione transferases, which have gained appreciation as major contributors for the metabolism of certain chemotypes (Guengerich, 1989). The S9 data set is, therefore, richer in content and provides researchers with an opportunity to stabilize compounds against both Phase I and II simultaneously. Human (gender pooled, 10 individuals) liver S9 fractions were purchased from BD Biosciences (San Jose, CA., USA). The incubation conditions were developed by using four commercial compounds, 7-EC, diclofenac, 4-nitrocatecal, and phenolphthalein, with known metabolic profiles (both Phase I and II metabolism). The S9 protein concentration and the cofactor concentrations were optimized to match the results of hepatocyte stability quantitatively. The Phase I and Phase II metabolites; 7-hydroxycoumarin (7-HC), 7-HC sulfate, 7-HC glucuronide, 4-hydroxydiclofenac (4-HD), 4-HD glucuronide, diclofenac acyl glucuronide, 4-nitrocatechol sulfate (4-NC sulfate), and phenolphthalein glucuronide were also monitored. The identity of each of the metabolites was assessed by comparison of their retention time and mass spectra with those of authentic standards. A cocktail of four activating cofactors was used in order to stimulate Phase I (NADPH), and Phase II (UDPGA, PAPS, GSH) metabolism. The final concentrations of NADPH, UDPGA, and GSH were 1, 0.5, and 2.5 mM, respectively, while that of PAPS was 0.05 mg/mL. The incubation conditions were developed by using four commercial compounds, 7-EC, diclofenac, 4-nitrocatecal, and phenolphthalein, with known metabolic profiles (both Phase I and II metabolism). The S9 protein concentration and the cofactor concentrations were optimized to match the results of hepatocyte stability, quantitatively. The Phase I and Phase II metabolites; 7-hydroxycoumarin (7-HC), 7-HC sulfate, 7-HC glucuronide, 4-hydroxydiclofenac (4-HD), 4-HD glucuronide, diclofenac acyl glucuronide, 4-nitrocatechol sulfate (4-NC sulfate), and phenolphthalein glucuronide were also monitored. The identity of each of the metabolites was assessed by comparison of their retention time and mass spectra with those of authentic standards. A cocktail of four activating cofactors was used to stimulate Phase I (NADPH) and Phase II (UDPGA, PAPS, GSH) metabolism. The final concentrations of NADPH, UDPGA, and GSH were 1, 0.5, and 2.5 mM, respectively, while that of PAPS was 0.05 mg/mL. Tris buffer was prepared as a 200 mM solution containing 2mM magnesium chloride (we included MgCl2 as a source for Mg+2 ions to stimulate CYP activity) in deionized water and adjusted with 1 M NaOH to pH 7.4. Stock reference solutions (7-EC as the positive control) and test compounds were prepared at 5 mM concentration in DMSO and then diluted to 0.3 mM with ACN prior to use. NADPH, UDPGA, and GSH solutions were prepared at 40, 20, and 2 mM, respectively, while PAPS was prepared at 2 mg/mL, all in tris buffer prior to mixing in a 1:1:1:1 ratio for use. S9 fraction was preincubated with a test compound for 5 minutes at 37°C in tris buffer, pH 7.4; the reactions were initiated by adding the cofactor mixture. At two time points, zero and sixty minutes, aliquots of the sample mixture were removed and quenched by the addition of two volumes of ice-cold 50:50 ACN:MeOH. The plate of quenched samples was then centrifuged at 4000*g* for 10 minutes to sediment the precipitated proteins before injection onto LC-MS/MS for analysis of the remaining parent compound. The concentration of the parent compound remaining was calculated by comparing peak areas. In addition, for comparison purposes, we also calculated *in vitro* half-lives for the compounds using the liver microsomal metabolic stability assay ($t_{1/2}$). The result of this analysis is shown in Table

2. Based on previous studies (Li et al., 2010), compounds with *in vitro* microsomal half-lives < 30 min are binned as unstable, and those with *in vitro* microsomal half-lives ≥ 30 min are binned as stable. High stability rates are normally associated with poor permeability (Li et al., 2003). Both Qx-294 and Qx-301 exhibited moderate and high permeability rates, respectively, which is consistent with their lower metabolic stability rates (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

Overall, completion of Major Task 1/ Subtask 1 supports the premise that Qx-294 and Qx-301 have a lower IC_{50} (IC_{50} = 15) 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* with no significant signs of toxicity.

Major Task 1/Subtask 2

Specific Objective: To conduct *In vivo* testing of Qx's top analogs using zebrafish to determine the concentration range of the analogs.

Key Outcomes: In zebrafish, *Danio rerio*, HCs are found within neuromasts of the lateral line, a superficial sensory organ that detect differential water movements (Montgomery et al., 2000). Like mammals' inner ear sensory epithelia, each neuromast contains sensory HCs and nonsensory SCs (Metcalfe et al., 1985; Raible and Kruse, 2000; Gompel et al., 2001). Lateral line HCs share structural, functional, and molecular similarities with those in the mammalian inner ear (Whitfield, 2002; Nicolson, 2005). On the other hand, the SCs are divided into two distinct support cell populations: *inner support cells*, situated underneath and adjacent to hair cells, and *mantle cells,* which form a ring of cells encircling the entire neuromast (Hardy et al., 2021).

Over the last several years, much work has gone into characterizing and establishing the zebrafish lateral line as a model for sensory hair cell regeneration: following neuromast HC death, Complete regeneration of HC numbers is typically achieved within 3 days through induction of proliferation and subsequent differentiation of inner SCs (Lopez-Schier and Hudspeth, 2006, Ma et al., 2008, Mackenzie and Raible, 2012, Wibowo et al., 2011). As in zebrafish, SC proliferation is vital for HC regeneration in mammals. Therefore, we sought to conduct *In vivo* testing of the Qx-294 and Qx-301 in 5dpf *Tg(brn3c:GFP)* zebrafish to determine the concentration range of the analogs required to stimulate SC proliferation and HC regeneration without signs of toxicity. Healthy 5 dpf zebrafish were randomly distributed into 24-well plates (10 larvae per well) and exposed to various concentrations of Qx-294 and Qx-301 (50, 100, 150, 200, 250, and 300 ug/mL) diluted in swimming (E3) medium for 24 hours. Control groups consisted of zebrafish treated with 0.1% DMSO and untreated animals. Of note, both compounds were dissolved in in DMSO at stock concentrations of 20mM and then diluted to their final concentrations in an E3 medium. No dead larvae were recorded at any concentration tested. Fish behavior (experimental and control) was recorded employing a ZebraLab & ZebraBox turnkey for PPI (Viewpoint Life Sciences). No changes in swimming ability between experimental and control groups were observed. Bright-field images of the larvae were captured using a fluorescence stereomicroscope (AXIO Zoom. V16, ZEISS, Oberkochen, Germany) 24 hours prior to fixation and continuation of experiments (Figure 2A, B). HCs were immunostained with otoferlin(HCS-1, Developmental Studies

Hybridoma Bank) and GFP (NB100-1614, Novus Biologicals) antibodies. Results were compared between treatment groups and assessed for neuromast and HC integrity (Figure 2C-H). Overall, no structural differences were observed between control and treatment groups.

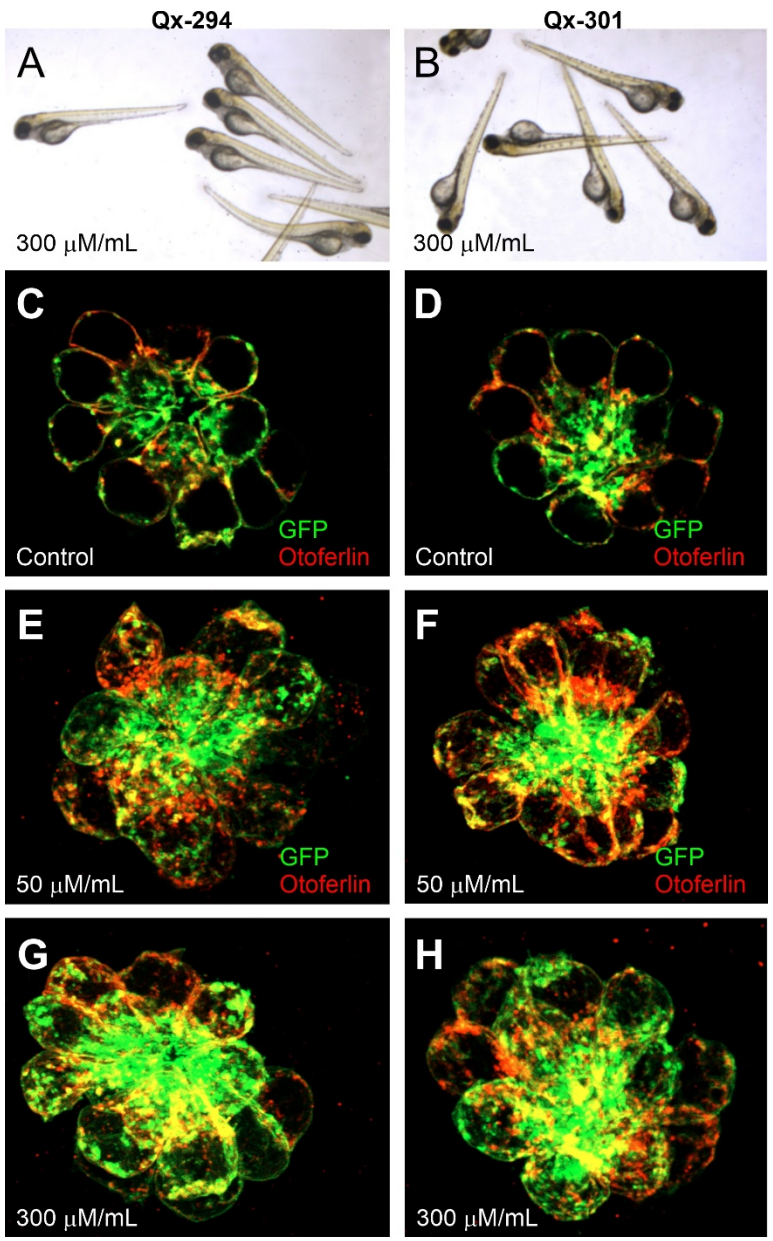


Figure 2: Increasing concentrations of Qx-294 and Qx-301 do not affect the integrity or survival of neuromast HCs *in vivo*. **A, B.** 5dpf *Tg(brn3c:GFP)* zebrafish treated with Qx-294 or Qx-301. **C-H.** Regardless of the drug concentration tested, no structural changes were observed on HCs' structure or neuromast anatomy. Given the lack of variability on the overall integrity of the HCs and neuromast structure, only images for the lowest and highest concentrations treatments are shown here (E-H).

Assessing the Effect of Qx-294 and Qx-301 on zebrafish neuromast: The GFP (*Tg(brn3c: GFP)* zebrafish larvae expressing HC membrane-bound GFP were used to evaluate the effect of Qx-291 and Qx-301 on HCs and SCs of the lateral line neuromasts. Healthy 5 dpf zebrafish were randomly distributed into 24-well plates (10 larvae per well) and exposed to various concentrations of Qx-294 and Qx-301 (50, 100, 150, 200, 250, and 300 ug/mL) diluted in swimming (E3) medium for 24 hours. After treatment, animals were transferred to E3 media for 30 min to recover and then fixed with 4% paraformaldehyde (PFA) overnight at 4 °C. Immunohistochemistry experiments were performed as previously described (Rocha-Sanchez et al., 2018) using otoferlin and GFP antibodies (data not shown but see Figure 2C-H). An arbitrary scoring protocol was used to rank the morphology of each neuromast after treatment as follows: 1 (normal rosette-like shape, HCs look normal, hair cell bundles are properly arranged), 2 (normal rosette-like shape, a few HCs appear to be missing, but hair cell bundles are properly arranged), 3 (normal rosette-like morphology but several HCs are gone and hair cell bundles look disturbed), 4 (the rosette-like shape is lost in certain areas, few HCs, and few disrupted hair cell bundles), 5 (No rosette-like shape, one or two disperse hair cells and no hair cell bundles). At least 25 animals per treatment were assessed. The assessment was performed double-blind and unbiased. Scores were expressed as percentages of the total of neuromasts analyzed per treatment (Figure 3A, B).

To examine the effect of Qx-294 and Qx-301 exposure in zebrafish HC survival, we performed terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. To detect potential apoptosis in *Tg(brn3c: GFP* lateral line neuromasts we employed the *in situ* cell death detection kit, TMR red (Roche), according to the manufacturer's instructions. Previous studies have shown that TUNEL assays performed after longer incubations led to TUNEL-negative HCs. To identify any potential false negative results, animals were incubated with Qx-294 and Qx-300 for 4, 6, 12, and 24 hours. This incubation approach was chosen to eliminate the chance of TUNEL-

negative HCs. Since no differences were observed between the different incubation periods and to keep the incubation times uniform, all subsequent experiments followed a 6-hour incubation period. Positive controls consisted of zebrafish incubated with 400 uM cisplatin for 6 hours. Compared to the positive control (figure 4A), no signs of apoptosis were observed in the neuromasts of zebrafish treated with Qx-294 (Figure 4B-E) or Qx-301 (Figure 4F-I) at any concentration.

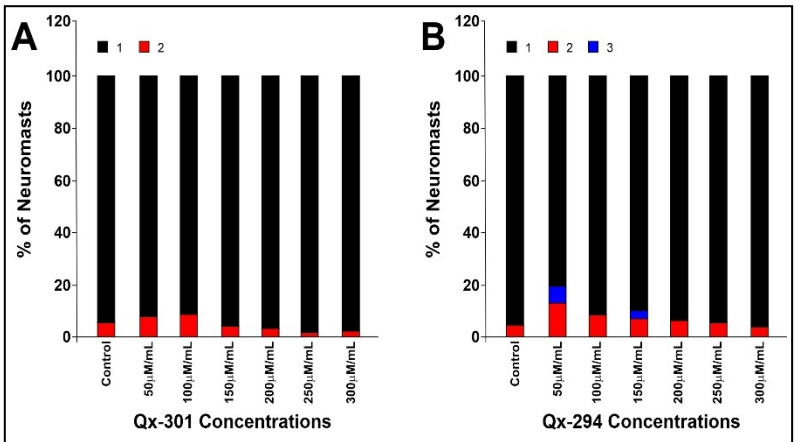


Figure 3: Scores for neuromast morphology (see text for details). **A, B**. Data were taken from at least 25 animals and 3 experiments runs. Except for a few HC loss, which was not statistically significant when compared to the control (vehicle-only)- treated samples, no changes to the overall integrity of the neuromasts and HCs were observed. Data were taken from at least 25 animals and 3 experiments runs.

Next, we tested Qx-294 and Qx-301 proliferative potential on zebrafish neuromasts. To that end, we performed cell proliferation with bromodeoxyuridine (BrdU) followed by BrdU immunohistochemistry, according to Rocha-Sanchez et al. (2018). Briefly, animals were incubated for 24 hours with different concentrations (50, 100, 150, 200, 250, and 300 ug/mL) of QX-294 or Qx-301 diluted in swimming (E3) media, followed by incubation with BrdU as previously described (Rocha-Sanchez et al., 2018). Controls (Fig 5A, B) consisted of vehicle (DMSO)-only treated fish. BrdU incorporation was analyzed by immunohistochemistry. Differences between compounds and between concentrations within each compound were tested by one-way ANOVA. Results were presented as mean ± SEM. Consistent with their potential effect on HC regeneration, BrdU-positive SCs were observed in neuromasts of fish treated with either Qx-294 or Qx-301 at all concentrations (Figure 5C-J). Of note, HCs were consistently BrdU-negative, regardless of the compound or the treatment concentration (Figure 5C-J). The number of proliferating cells per compound and concentrations were assessed by counting BrdU-positive cells in confocal stacks of imaged neuromasts (Figure 5K). For each treatment, 25 animals were used, and the experiments were repeated three times. Three neuromasts per fish

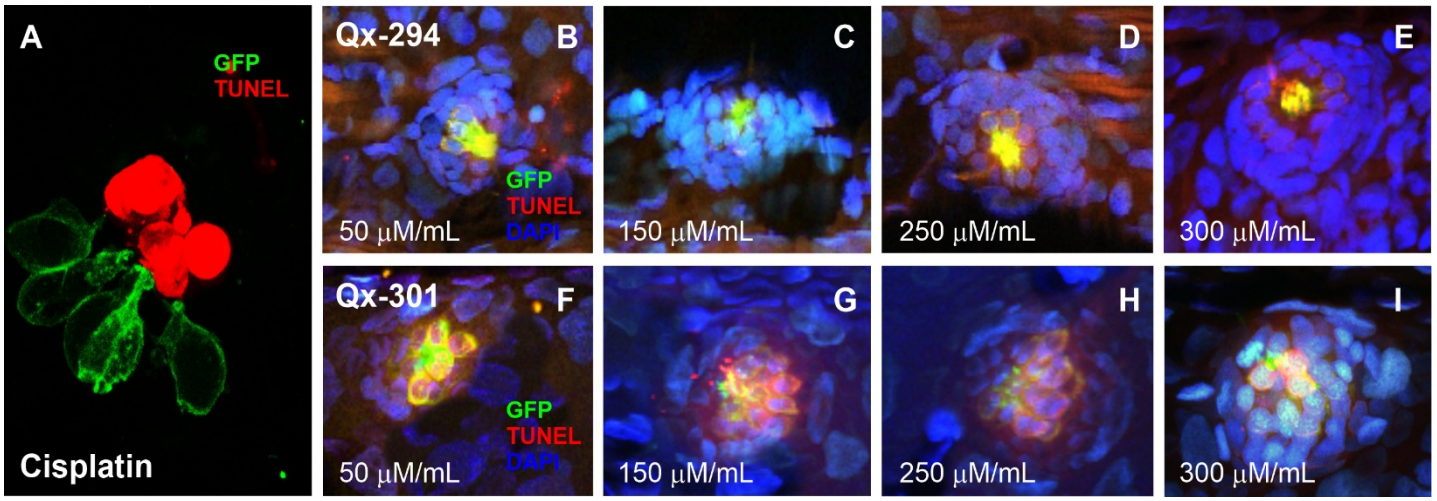


Figure 4: Qx-294 and Qx-301 have no cytotoxic effect on neuromast cells. TUNEL assay (red) was performed in zebrafish incubated with vehicle (negative control), 400 µM cisplatin (positive control) or different concentrations of Qx-294 and Qx-301. **A.** Consistent with its known cytotoxic effects, extensive cell death was observed on cisplatin-treated animals. **B-I.** Regardless of the drug or concentration, no differences on TUNEL results were observed on experimental animals as well as between experimental animals and negative control. Due to the lack of variability on the experimental results, only four of the six concentrations tested are shown for each compound. Animals were also immunostained for GFP (green). Given the lack of variation on the results, not all treatment concentrations are shown here.

were assessed. Qx-294 and Qx-301-treated fish displayed a significantly higher number of proliferating cells than the control groups ($P < 0.05$; Figure 5K). However, when compared, the number of BrdU-positive SCs was significantly higher on Qx-301- than on Qx-294-treated neuromasts ($P < 0.05$; Figure 5C-J).

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 (Figure 6A-D). FM1-43FX (Thermo Fisher) uptake experiments were performed as previously described (Rocha-Sanchez et al., 2018). Animals were incubated with E3 alone (control) or in the presence of Qx-294 or Qx-301 both at 300 µM for 7 hours and then immediately exposed to 3 µM of FM1-43FX for 40 sec or animals were transferred to a fresh E3 solution for 1 hour and then exposed to the dye. Fluorescence incorporated was quantified according to (Rocha-Sanchez et al. (2018) using ImageJ. Results summarized in Figure 6A-C show that rapid dye entry into the HCs was comparable between control and experimental groups for both compounds. No visual differences were observed in FM1-43 uptake on fish transferred to fresh media before treatment with FM1-43 (data not shown). Quantification of the fluorescence intensity incorporated by the neuromasts did not show any significant differences between treatment or control groups (Fig. 6D), indicating that neither Qx-294 nor Qx-301 block the mechanotransduction channels and supporting the premise that Qx-induced, newly generated HCs in the zebrafish neuromasts are functional.

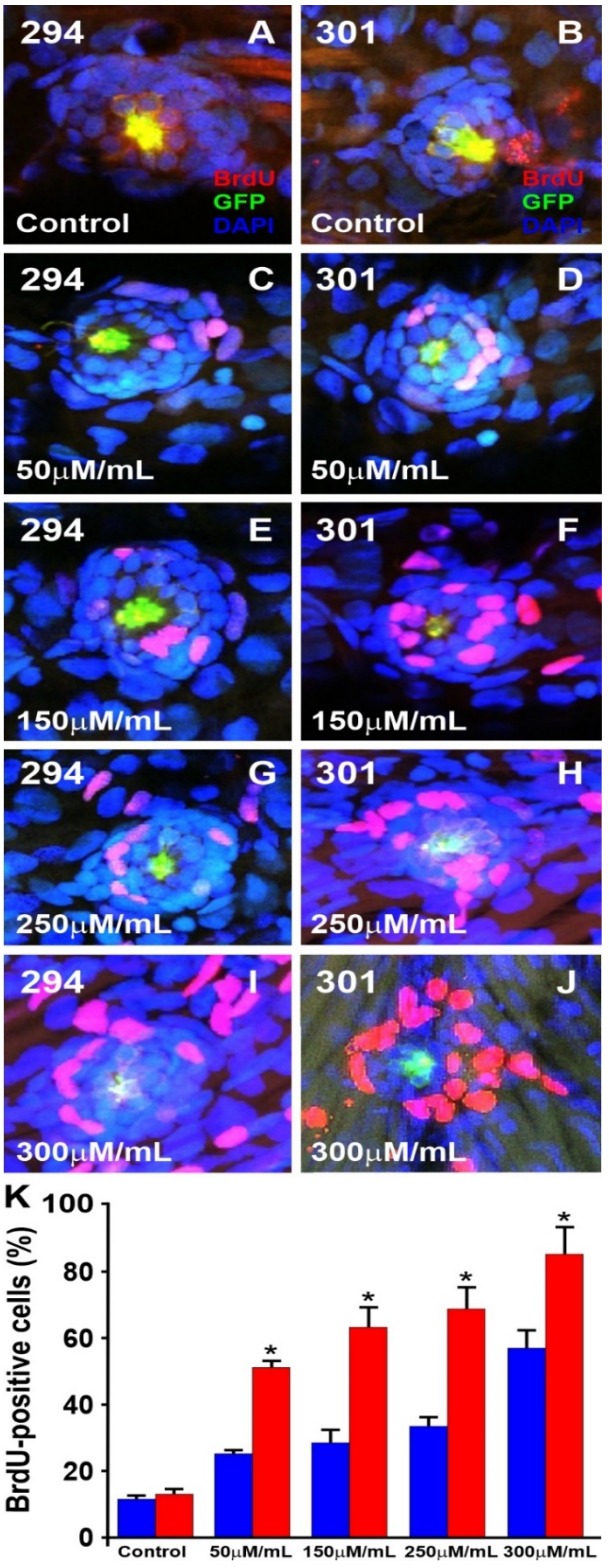


Figure 5: Proliferation assay following Qx-294 or Qx-301 treatment. **A, B.** No BrdU-labelling (red) was seen in the neuromast's cells of control animals. **C-H.** BrdU-positive cells were seen in the SCs surrounding HCs (GFP/green). **H.** The percentage of BrdU-positive supporting cells per neuromast was calculated for each treatment and represented as mean +/− SEM. One-way ANOVA, *p < 0.05.

Major Task 1/Subtask 3

Specific Objective: To test Qx top analogs' potency (IC_{50}) and toxicity (LD_{50}) in mouse cochlear explants.

Key Outcomes: To prepare cochlear organotypic explant culture, cochleae of 3 days old postnatal C57BL/6 mice were dissected and the spiral ganglion, Reissner's membrane, and the stria vascularis were carefully removed. Cochlear explants were plated onto 35 mm^2 dishes coated dishes coated with poly-L-lysine (Sigma-Aldrich, St. Louis, MO) and cultured in DMEM/F12 medium with N2/B27 supplement (Invitrogen). After the explants were attached to the dishes, the culture medium was removed and replaced with a medium containing Qx-294 and Qx-301 diluted to 300 ug/mL and

incubated for 24 hours. The concentration's choice was supported by our previous *in vitro* and zebrafish experiments. After treatment, the explants were fixed and stained with myosin VIIa antibody to identify the HCs (Figure 7A-C). The numbers of surviving HCs across the three turns of the organ of Corti were counted (Figure 7D). One-way ANOVA was used to compare the difference in mean cell numbers between and within groups. All values are presented as the mean ± standard error. *P* Values less than .05 were considered statistically significant. IC_{50s} were calculated by non-linear regression of data normalized by the percentage change of the controls (Figure 7D). The number of technical and biological replicates is indicated in Figure Legends.

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 hours incubation period (Figure 7A-C) and at a much higher rate than in the control group (Figure 7D). Fine-tunning of the BrdU treatment (e.g., BrdU pulse-chase) to assess the timing of cell cycling, use of younger animals to start explant cultures, and/or adjustments to the drugs' concentration will be attempted to improve the outcome.

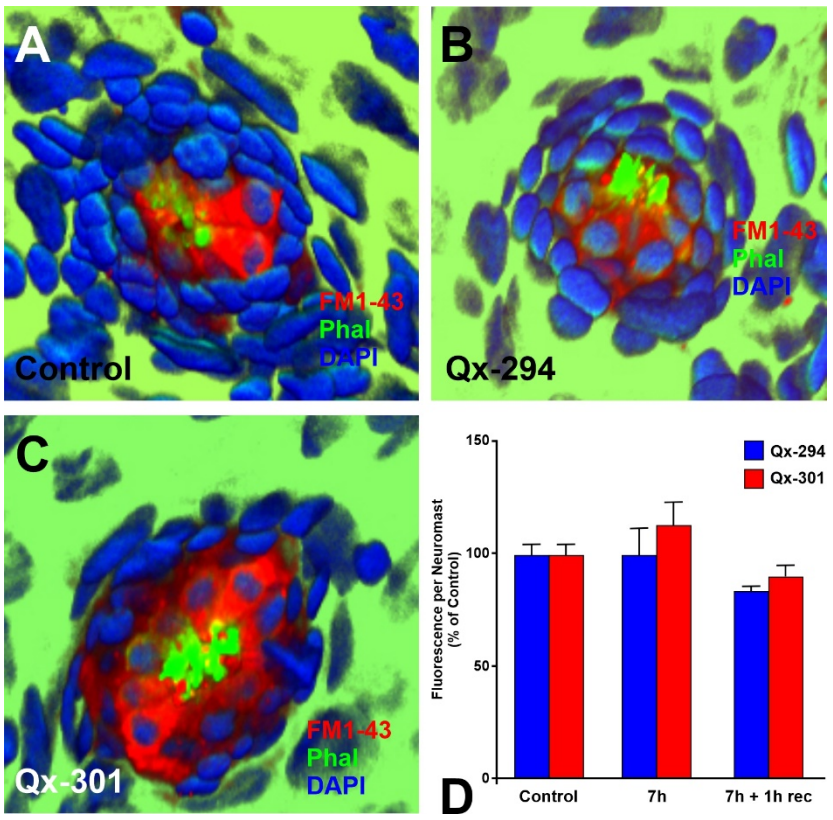


Figure 6: Treatment with Qx-294 or Qx-301 does not abolish mechanotransduction channel activity. 5dpf wild-type larvae were incubated in E3 media (**A**). Animals were incubated on media containing Qx-294 or Qx-301 300 µM for 7 hours and immediately assayed for FM1-43 uptake (**B, C**). alternatively, following incubation with compounds, animals were recovered for 1 hour and then treated with the dye (data not shown). Quantification of the fluorescent intensity per neuromast (**D**) was expressed as a percentage of controls. No significant differences were observed between control and treated animals (unpaired Student's t-test).

Overall, completion of Major Task 1/ Subtasks 1, 2 and 3 supports the premise that:

1. Qx-294 and Qx-301 *in vivo* treatment leads to proliferation of mantel cells and generation of extra HCs in zebrafish neuromast and cochlear explants.

2. Both compounds' performance in cochlear explants resembles that of HEI-OC1 cells, as confirmed by the presence of supernumerary HCs.

3. Consistent with the *in vitro* studies, no signs of cell death were observed on zebrafish or cochlear explants treated with either compound at any concentration tested. Yet, proliferating cells were observed zebrafish treated with either drug. Likewise, supernumerary cells were observed in cochlear explants treated with Qx-301 at the highest concentration tested.

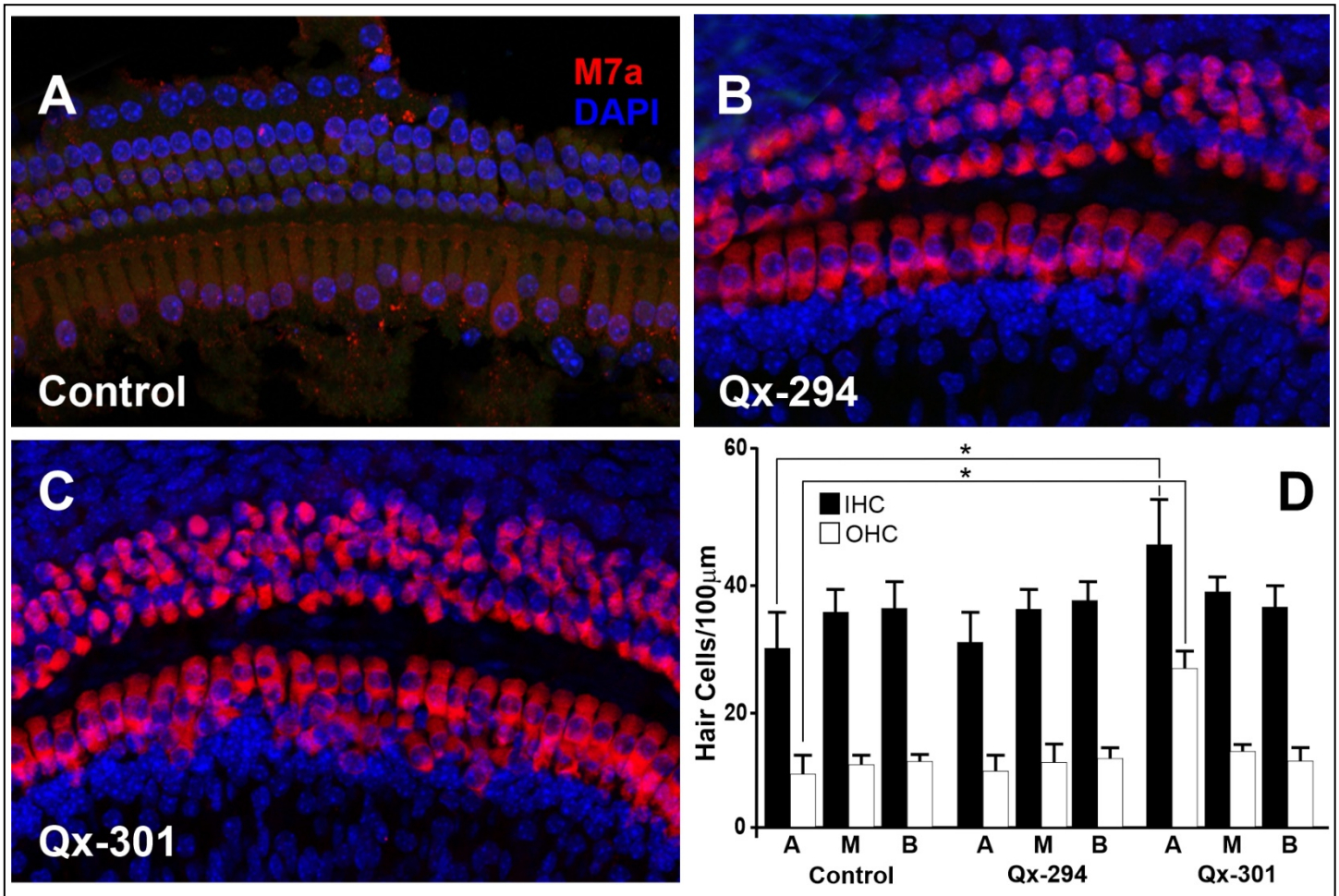


Figure 7: The effect of the two new compounds in cochlear explants. **A-C.** Confocal images of the apical, middle, and basal turns in cochlear explant cultures containing **(A)** 0.1% DMSO (control), **(B)** 300 mg/Kg Qx-294, and **(C)** 300 mg/Kg Qx301 for 24 hours. No cytotoxicity to the cochlear 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 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 arrangment of the OHCs. Nevertheless, supernumeraty IHCs were smostly located outside the normal IHC axis in the inner phalangeal and inner border cells' region. 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.

Major Task 1/Subtask 4

Specific Objective: To conduct *In vivo* Pharmacodynamics (PD) and Pharmacokinetics (PK) assessments.

Key Outcomes: This set of experiments just started. Results are too preliminary to be presented at this time.

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

Experiments towards Specific Aim 2 have recently started. Results are too preliminary to be presented at this time.

We are currently organizing the results for publication. A spin-off of the present study, we have contributed to the characterization of a novel antibiotic, which shows no ototoxic effects *in vitro* and *in vivo* **(Appendix 1)**.

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3.3 Opportunities for training

Nothing to report

3.4 Disseminating the results to the community of interest

Nothing to report

3.5 Plans for next reporting period

In consequence of the pandemic shutdown, limited access to the different research facilities, and limited access to research supplies and reagents due to supply chain issues, the proposed work is delayed. We submitted a request for non-cost extension and have ben granted one more year to complete the study. That time will suffice to complete the proposed work and submit the results for publication. Specifically, we have successfully completed all the in-vitro studies, modified the original Qx formulation, screened 70 new analogs, and identified two new compounds (Qx-294 and Qx-301) with IC_{50} lower than the original compound. During our next reporting period we plan to:

- To conduct *In vivo* Pharmacodynamics (PD) and Pharmacokinetics (PK) assessments in mice treated with the two new compounds.

- 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

4. 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

5. 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

6. 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*. *Under revision*.

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

Nothing to report

6.1.3 Other publications, conference papers, and presentations

Cunningham K., Calisto L., **Rocha-Sanchez SM**, North EJ. Design, Synthesis and Biological Evaluation of Novel Antimycobacterial Acetamides, *Creighton University Saint Albert Research Day.* April 2022.

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

7. 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 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 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:	Kamil Ikramullah
Project Role:	Work-Study Student
Research Identifier:	None
Nearest person month worked:	6
Contribution to project:	Kamil assisted on immunohistochemistry, tissue dissection, and *in vitro* drug treatments associated with the study
Funding Support:	This award

Name:	Menghini, Alexis R
Project Role:	Work-Study Student
Research Identifier:	None
Nearest person month worked:	6
Contribution to project:	Alexis assisted on immunohistochemistry, tissue dissection, and *in vivo* 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

8. 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 2

Appendices

Appendix 1

From:	Journal of Medicinal Chemistry
To:	North, Jeffrey
Cc:	Bhattarai, Pankaj; Hegde, Pooja V; Wei.Li@colostate.edu; Prathipati, Pavan Kumar; casey.stevens@ou.edu; Lyang403@gatech.edu; Zhou, Hinman; Pandya, Amitkumar N; Cunningham, Katherine; Grissom, Jenny N; Roman, Mariaelena; Sowards, Melanie A; Calisto, Lily E; Destache, Christopher J; Rocha-Sanchez, Sonia; gumbart@physics.gatech.edu; elenaz@ou.edu; Mary.Jackson@colostate.edu; North, Jeffrey
Subject:	Manuscript jm-2022-00352k.R1 assigned to Editor
Date:	Saturday, August 6, 2022 2:04:13 AM

06-Aug-2022

Journal: Journal of Medicinal Chemistry
Manuscript ID: jm-2022-00352k.R1
Title: "Structural Determinants of Indole-2-carboxamides: Identification of Lead Acetamides with Pan Anti-mycobacterial Activity"
Author(s): Bhattarai, Pankaj; Hegde, Pooja; Li, Wei; Prathipati, Pavan; Stevens, Casey; Yang, Lixinhao; Zhou, Hinman; Pandya, Amit; Cunningham, Katie; Grissom, Jenny; Roman Sotelo, Mariaelena; Sowards, Melanie; Calisto, Lilian; Destache, Christopher; Rocha-Sanchez, Sonia; Gumbart, James; Zgurskaya, Helen; Jackson, Mary; North, E. Jeffrey
Manuscript Status: Associate Editor Assigned

Dear Dr. North:

Your revised manuscript entitled "Structural Determinants of Indole-2-carboxamides: Identification of Lead Acetamides with Pan Anti-mycobacterial Activity" has been received.

Please address all future correspondence regarding this manuscript to the below editor:

Dr. Lori Ferrins
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Topic Editor
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Appendix 2

Pharmacologic Regulation of Auditory Hair Cell Regeneration

W81XWH-20-1-0789
RH190008

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

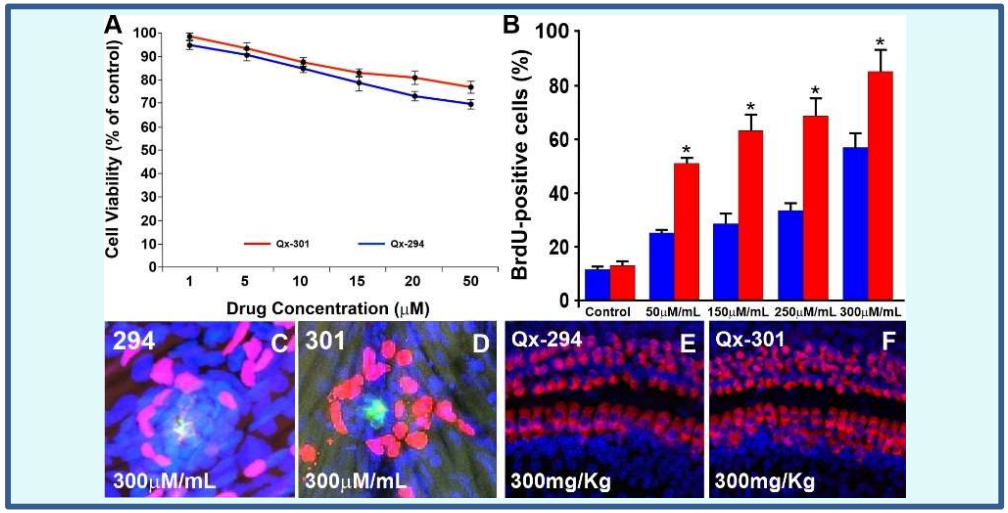
Study/Product Aim(s)

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

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 the zebrafish neuromast without signs of apoptosis. Overall, the results support the continuation of the studies in a mammalian (mouse) system.



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 **(B, C)** and inner ear **(E, F)** Supporting Cells.

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: (09/27/2022)

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 – In progress
- ☐ Randomized PK assessment of best oral dose determined on subtask 1, at 0.5, 1, 2, 8- and 24-hours post administration - In progress

Comments and Challenges: Supply chain issues and shipping/delivery delays of reagents and supplies have greatly affected our timeline. We are currently working towards completion of all aims and organizing the data for publication.