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TITLE**: Exploring the Beneficial Effects of Quinoline Derivatives After Noise-Induced Damage**

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CONTRACTING ORGANIZATION: **Creighton University, Omaha, NE**

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

More than 300 million people worldwide suffer from noise-induced hearing loss (NIHL), with members of the military service being a particular susceptible group due to their, sometimes unpredictable, exposure to intense acoustic trauma. Despite the enormous social and economic impact of NIHL there are currently no therapies to prevent or alleviate this condition. Published work and preliminary studies from our laboratory suggest that quinoline derivatives have the potential to protect against drug- and noise-induced hearing loss. The advantage of working with quinoline derivatives is that they have an excellent malleable structure for medicinal chemistry modifications, most of them are water soluble and stable at room temperature. Moreover, quinoline derivatives have been approved by the FDA for their use in the pharmaceutical and food industries, which could expedite their development as therapeutic compounds to treat hearing loss. The goal of this award is to synthesize, screen and identify quinoline derivatives that will protect against NIHL. During the first year of this proposal we synthesized 70 analogs that were then, tested in a zebrafish model for hair cell excitotoxicity. Out of the 70, 31 protected the hair cells from the kainic acid toxic effect. Of those 31, we have currently tested 7 for protection of the hair cell ribbon synapses. We found that only two, Qx-1 and Qx-62, prevented the loss of ribbon synapses. We are currently testing the rest of the 31 compounds to assess whether they can also prevent from kainic-acid induced synaptopathy. These results are significant not only because we were able to identify two compounds that have the potential to protect hair cells and synapses from excitotoxic damage but also because we will be able to move forward with, at least, those two compounds and test them in a mouse model for NIHL.

15. SUBJECT TERMS

None listed.

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSID**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**	Unclassified	14	**19b. TELEPHONE NUMBER** *(include area code)*
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1. Introduction

According to the Centers for Disease Control and Prevention, more than 22 million Americans are exposed to occupational hearing loss each year [1]. Among them, members of the military service are a particularly susceptible group that can develop noise-induced hearing loss (NIHL) due to intense acoustic trauma from artillery gunfire and explosives[2] [3]. NIHL is associated with the irreversible loss of hair cells (HCs) and spiral ganglion neurons as a result of the generation of reactive oxygen species (ROS) by the increasing metabolic activity[4]. Many studies have demonstrated the existence of inflammatory response after noise overstimulation that involves the up-regulation of pro-inflammatory molecules as a result of nuclear factor kB (NF-kB) pathway activation[4],[5]. Unfortunately, despite the enormous impact of NIHL, there are no therapeutic interventions that may help to prevent or alleviate this problem. Most candidate compounds currently in pre-clinical and clinical trials are related to antioxidants, vitamins, and glutathione metabolism and their effectiveness remain unclear[4] [6]. Previous work from our laboratory[7,8] supports the therapeutic potential of quinoline derivatives to treat ototoxin- and possibly NIHL. Unlike some agents currently in pre-clinical or clinical trials, which act at later stages during ROS generation, when irreversible oxidative damage to the auditory system may have already happened[4-6], quinoline derivatives act upstream of ROS, blocking the activation of the NF-kB pathway[6, 8-14]. Therefore, by blocking NF-kB activation, quinoline-derivatives help the cells to recover from the ototoxic insult before any irreversible damage takes place. The study of quinoline derivatives has become a subject of interest in recent years due to their wide variety of biological activities as well as therapeutic applications[13-15]. Moreover, certain quinoline derivatives have been approved by the FDA as chemical compounds for use in the pharmaceutical and food industries[16], which would expedite their developmental phase for use in the auditory system as repurposed compounds[17]. Based on this information we decided to investigate the therapeutic potential of quinoline derivatives against NIHL. For this purpose, we synthesized and screened 70 quinoline derivatives in a zebrafish model that mimics noise-induced excitotoxicity. Thirty-one of these compounds protected hair cells from dying. Moreover, we tested seven compounds out of the 31 and found that two preserved hair cell ribbons synapses. Overall, we have currently identified two compounds that have the potential to protect against NIHL. Future experiments will be aimed at testing them in a mouse model for noise damage. NF-kB regulation will be also assessed in the presence of these protective compounds.

2. Keywords

Noise-induced hearing loss, quinoxaline, NF-kB pathway, synaptopathy, zebrafish drug screening.

3. Accomplishments

MAJOR GOALS OF THE PROJECT

<u>Specific Aim 1</u>: Assess the specific therapeutic effect of quinoline derivatives against noise trauma.

Major Task 1: Procurement and screening of quinoline derivatives in zebrafish

Subtask 1: 3D-modeling and quinoline derivatives synthesis or acquisition.

1) Modelling of quinoline derivatives and analysis of the interaction with IKKb active site.

   Completed October 2020

2) Synthesis of quinoline derivatives or acquisition through commercial sources.

Initiated in October 2020 and completed in December 2020.

Subtask 2: Test of quinoline derivatives in a zebrafish model for noise.

1) Quinoline derivatives will be tested in a zebrafish model for acoustic trauma.

   100% completed

2) Immunohistochemistry processing.

   100% completed

3) Quantification and data processing and analysis (i.e., number of hair cells, ribbon synapses, neuronal terminals, etc.).

   50% completion

Major Task 2: Test the top five quinoline analogs in a mouse model for hearing loss.

50% completion

<u>Specific Aim 2</u>: Assess the involvement of the top lead quinoline derivatives in the NF-kB pathway

Major Task 3: Test the top quinoline analogs for NF-kB pathway inhibition and identification of their corresponding molecular target.

Subtask 1: Inhibition of NF-□B activation by quinoline derivatives after pharmacologically induced "noise" trauma in zebrafish.

1) Incubation of the *Tg*(*NFKB:EGFP*) line with the quinoline derivatives after the induction of excitotoxicity.

   100% completed

2) Immunohistochemistry processing.

   100% completed

3) Evaluation of NF-kB inhibition by quantification of GFP expression and fluorescent intensity in hair cells.

   25% completed.

Subtask 2: Identification of quinoline derivatives' molecular target in hair cells by the generation of zebrafish morphants.

1) Design, ordering and synthesis of the morpholino suspensions.

   100% completed

2) Design and synthesis of the copy RNA specific to each of the morpholino's molecular target.

   100% completed

3) Generation of the morphants in the *Tg*(*NFKB:EGFP*) and *Tg(brn3c:GFP)* fish lines.

   25% completed

4) Incubation of the *Tg*(*NFKB:EGFP*) and *Tg(brn3c:GFP)* lines with the quinoline derivatives after the induction of excitotoxicity.

   25% completed

5) Immunohistochemistry processing.

   25% completed

6) Evaluation of NF-kB inhibition by quantification of GFP fluorescent intensity, HC number and neuronal innervation.

   Samples have not been processed.

Subtask 3: Confirmation of NF-kB pathway inhibition in a mouse model for noise-induced damage.

The studies in mice have not been initiated.

ACCOMPLISHMENTS UNDER THESE GOALS.

1) Major activities:

The top five quinoline derivatives have been identified and tested in a noise mouse model that results in synaptopathy. Auditory brainstem responses (ABRs) and distortion product otoacoustic emissions (DPOAEs) have been assess before the noise and at different time points post-noise. The organs of Corti from the treated animals have been isolated and stained for neuronal markers. We haven't imaged all the groups yet.

Additionally, the NF-kB:GFP zebrafish line has been used to assess the quinoline derivatives mechanism of action. This has been finalized and we are currently performing the fluorescent quantification.

2) Specific objectives:

Specific Aim 1: Assess the specific therapeutic effect of quinoline derivatives against noise trauma. The goal is to expedite the identification of quinoline analogs that will promote recovery and/or repair of the HCs and their associated neuronal terminals after acoustic trauma. For this purpose, the following experiments were designed: **1)** computational 3D modeling and synthesis of quinoline derivatives by medicinal chemistry; **2)** screening in a zebrafish model that mimics noise-induced trauma, and **3)** test the top five lead candidates in CBA/CaJ mice.
Specific Aim 2. Assess the involvement of quinoline derivatives in the NF-kB pathway. Since the NF-kB pathway can be activated by acoustic trauma, we want to assess whether quinoline analogs are involved in the regulation of this pathway in HCs. For this purpose, the following experiments were designed: **1)** testing the quinoline derivative for NF-kB pathway inhibition in the zebrafish transgenic line *Tg*(*NFKB:EGFP*); **2)** identification of the NF-kB molecular targets by morpholino knockdown in zebrafish, and **3)** assessment of the NF-kB pathway activation after noise exposure in P45 CBA/CaJ mouse inner ear by immunohistochemistry and immunoblot experiments.

3) Significant results or key outcomes,

We have tested the top five derivatives against a synaptopathy model (94 dB SPL, 8-16 kHz band noise for 2 hours). ABRs and DPOAEs were tested before noise exposure (baseline), and at days

1, 7 and 21 post-noise. **Figure 1** show the results obtained with two of the quinoline derivatives, Qx23 and Qx62. These results suggest that while Qx62 fully portects against noise, Qx23 does not.

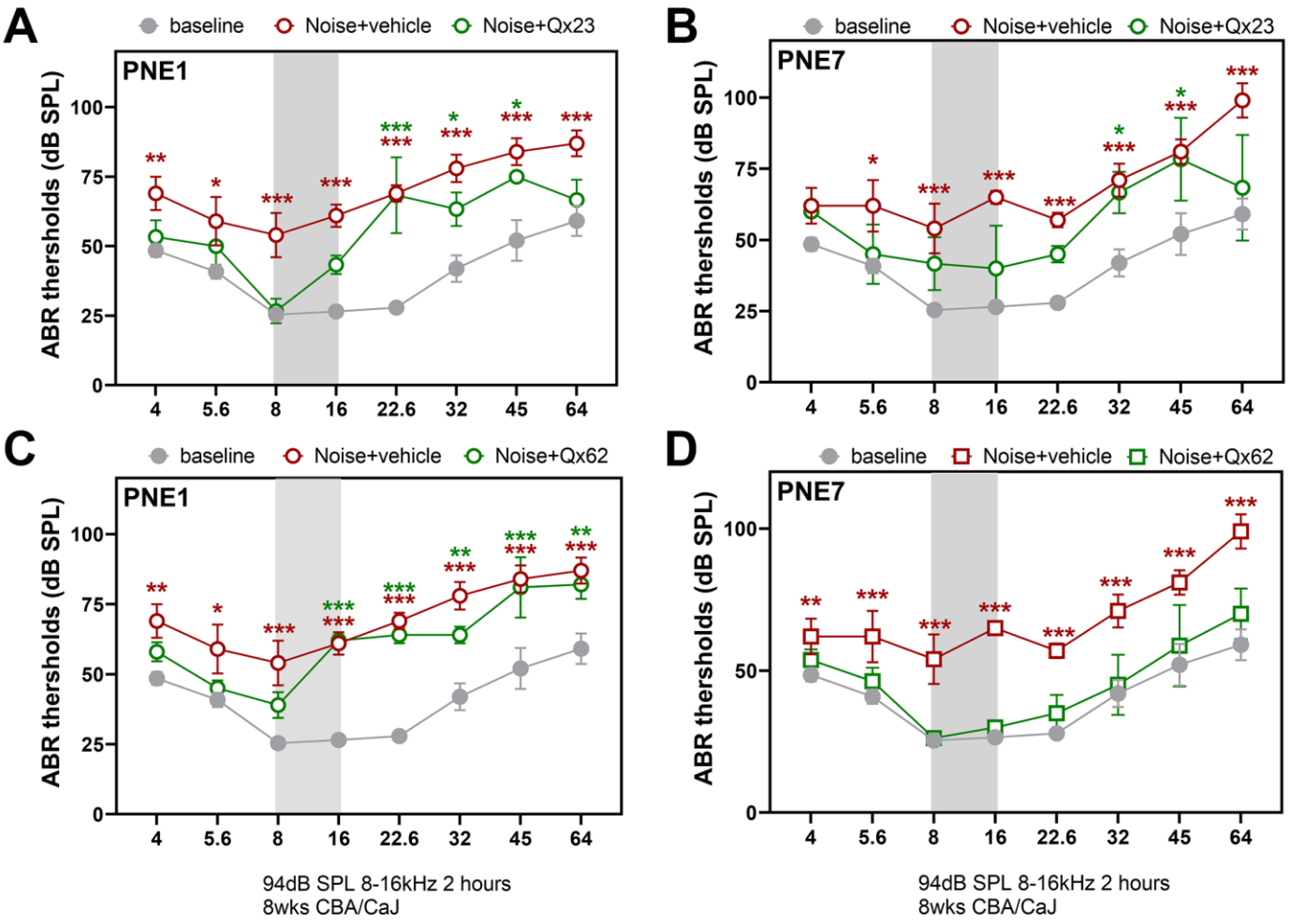

Figure 1. 7-8 weeks old CBA/CaJ were exposed to noise (94 dB SPL, 8-16 kHz band noise for 2 hours) and immediately intraperitoneally injected with 25 mg/kg b.w. of Qx23 (**A-B**), Qx62 (**C-D**) or corn oil (vehicle). Hearing tests were performed before noise exposure, 24 hrs after noise exposure and 7 days post-noise. Results are expressed as mean+/-SEM. Statistical analysis: Two-way ANOVA, *P<0.05, **P<0.01 and ***P<0.001 *versus* baseline (analysis is color coded)

We are currently performing histology experiments in all the groups so we will be able to quantify the number of hair cells and the number of pre-synaptic markers. As an example, Figs. 2-5 show representative images of animals treated with Qx23 and exposed to noise. These results confirm was we observed in our hearing tests (**Fig. 1**): After 7 days post noise, the pre-synaptic ribbons are still reduced in the noise-only groups for all the frequencies. Conversely, when animals were treated with Qx23, we observed a preservation of the pre-synaptic ribbons at all the frequencies except 32 kHz. These results much the lack of recovery in the ABR thresholds at 32kHz.

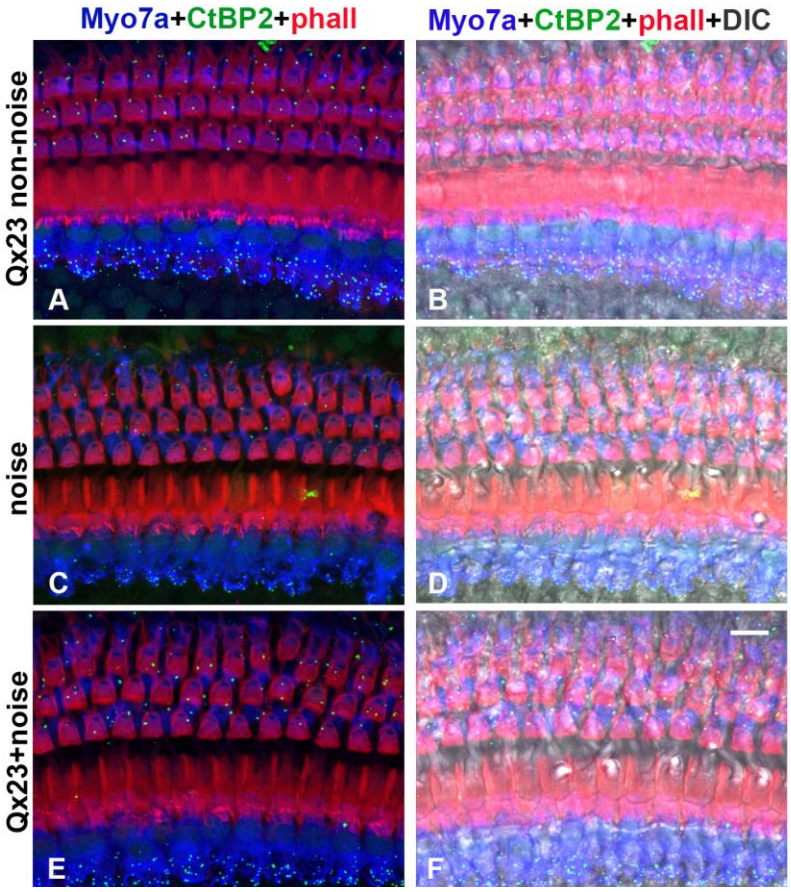

Figure 2. Representative confocal images of the 8 kHz region of organs of Corti from animals treated with Qx23 alone (25mg/kg b.w.) (**A-B**), noise (94dB SPL 8-16kHz, 2 hours) (**C-D**) and Qx23+noise (**E-F**). tissue was immunostained with the hair cell marker myosin 7A (blue) and the pre-synaptic marker CtBP2 (green). Phalloidin was used for F-actin (red). Scale bar=10 um.

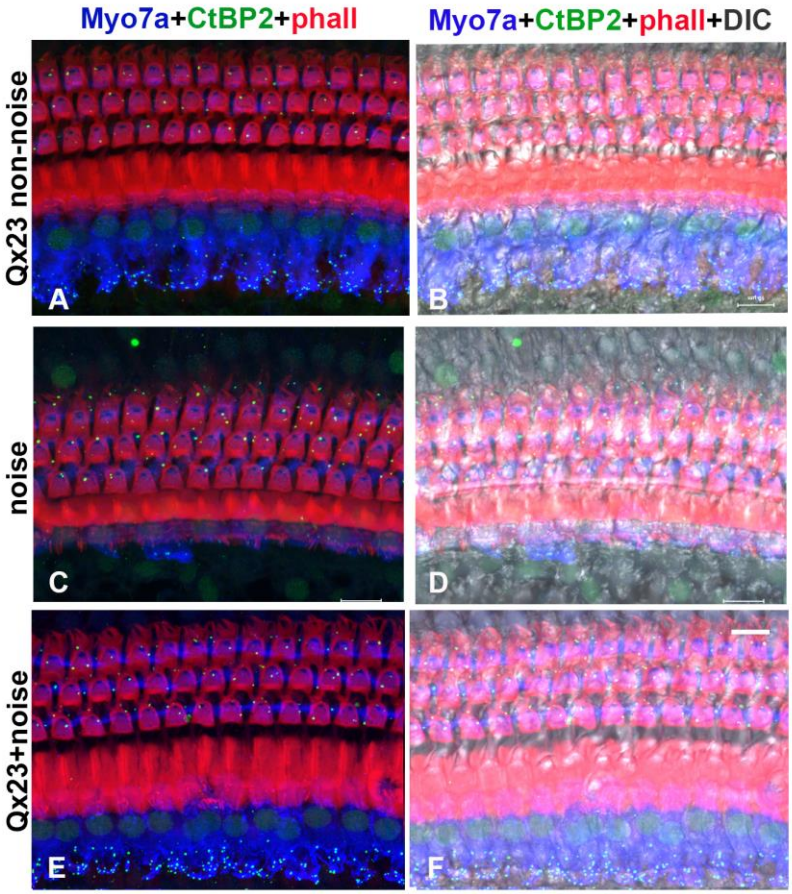

Figure 3. Representative confocal images of the 16 kHz region of organs of Corti from animals treated with Qx23 alone (25mg/kg b.w.) (**A-B**), noise (94dB SPL 8-16kHz, 2 hours) (**C-D**) and Qx23+noise (**E-F**). tissue was immunostained with the hair cell marker myosin 7A (blue) and the pre-synaptic marker CtBP2 (green). Phalloidin was used for F-actin (red). Scale bar=10 um.

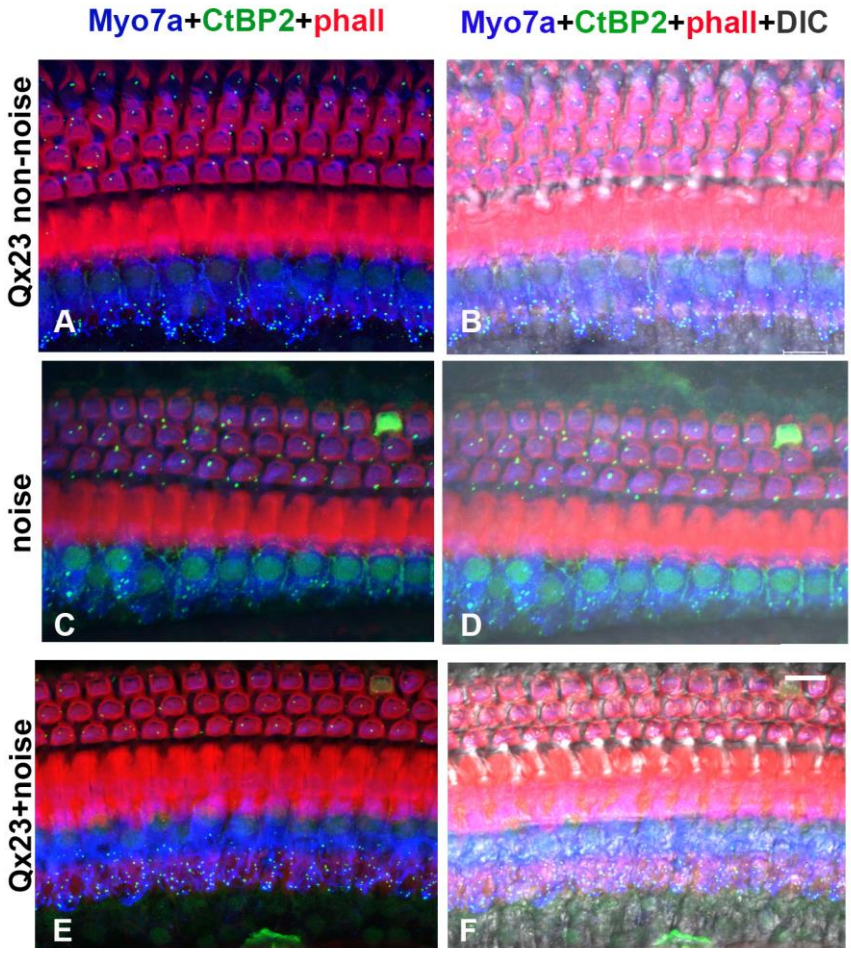

Figure 4. Representative confocal images of the 22.6 kHz region of organs of Corti from animals treated with Qx23 alone (25mg/kg b.w.) (**A-B**), noise (94dB SPL 8-16kHz, 2 hours) (**C-D**) and Qx23+noise (**E-F**). tissue was immunostained with the hair cell marker myosin 7A (blue) and the pre-synaptic marker CtBP2 (green). Phalloidin was used for F-actin (red). Scale bar=10 um.

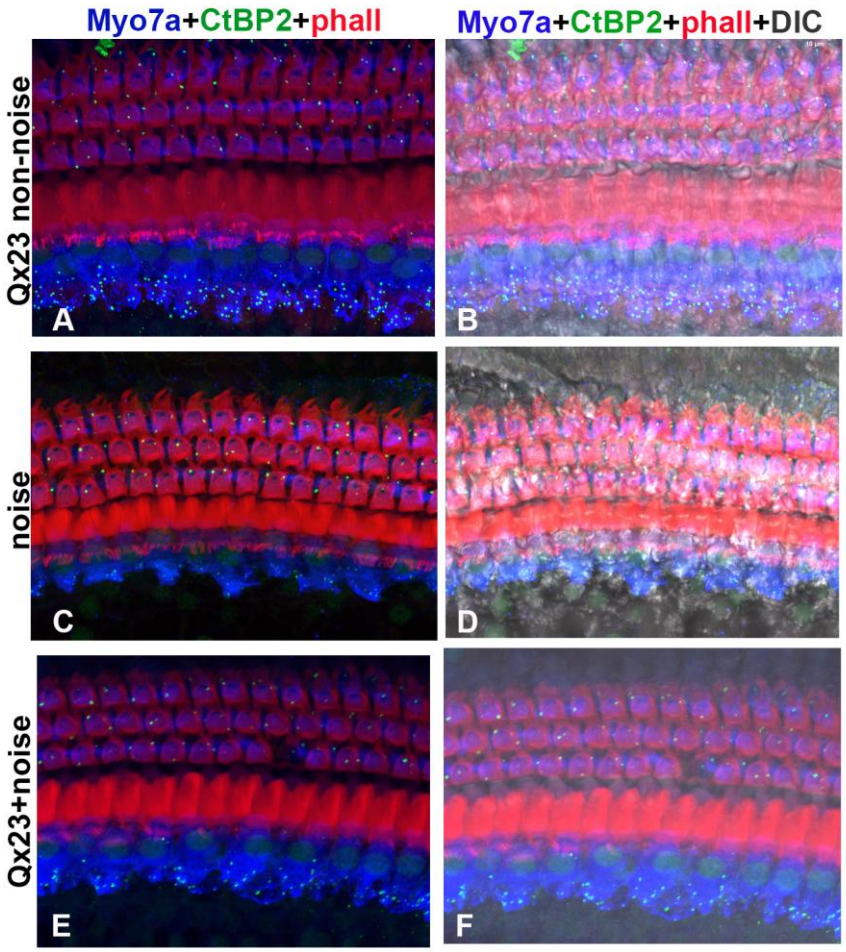

Figure 5. Representative confocal images of the 32 kHz region of organs of Corti from animals treated with Qx23 alone (25mg/kg b.w.) (**A-B**), noise (94dB SPL 8-16kHz, 2 hours) (**C-D**) and Qx23+noise (**E-F**). tissue was immunostained with the hair cell marker myosin 7A (blue) and the pre-synaptic marker CtBP2 (green). Phalloidin was used for F-actin (red). Scale bar=10 um.

The regulation of NF-kB pathway was assessed in the *Tg*(*NFKB:EGFP*) zebrafish line. For this purpose, 6 dpf animals were incubated with KA 300 µM for 1 hour followed by incubation with the quinoline derivatives for an additional 2 hours. Animals were fixed and immunostained for GFP (used as a proxy for NF-kB activation), otoferlin (hair cell marker), and p65 (NF-kB transcription factor that translocates to the nucleus when the pathway is activated). These samples are currently being processed.

Our findings are significant because we have been able to identify at least one compound (out of five) that can protect hair cells and ribbon synapses from synaptopathy.

Opportunities for training and professional development.

Nothing to report

Dissemination to communities of interest.

The results were presented at the Association for Research in Otolaryngology Midwinter Meeting held virtual during February 2022 and at the Molecular Biology of Hearing and Deafness, April 2022.

We have also presented part of our results at the Biomedical Sciences Department Seminar Series at Creighton University.

Plans for the next reporting period.

We are currently finishing with all the experiments involving zebrafish signaling cascade and we expect this work will be finished by September 2023.

During the rest of 2023, we will be testing these top 5 candidates in mouse models for noise-induced sensorineural hearing loss. We are also planning to assess whether the NF-kB pathway is inhibited by the quinoline derivatives in the organ of Corti.

4. Impact

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

The characterization of quinoline derivatives for protection against excitotoxic damage will likely make an impact in the search for therapies against NIHL. The fast screening in a zebrafish model that mimics noise damage followed by the confirmation of their protective effect in mice, will help to expedite the identification of small molecules for protection against NIHL in humans. Moreover, the initial selection of the best quinoline derivatives will set the foundations for additional chemical modifications to improve the potency and efficacy of the derivatives, leading to compounds with minimum side effects.

Impact on other disciplines

Nothing to report

Impact on technology transfer

Nothing to report

Impact on society beyond science and technology.

The impact on society will be very clear. The identification of quinoline derivatives that can protect against NIHL will improve the quality of life of those suffering from this condition and at the same time will reduce the economic burden associated with, for example, the used of hearing aids and alternative aid-technologies, counseling, etc.

5. Changes/Problems

We had a few minor problems. Our zebrafish NF-kB reporter line got contaminated with other lines containing a different genetic background. This is very common since transgenic fish are normally outcross with wild type lines and it is easy to make mistakes between females and males when they are put back into the system after breeding. To resolve this issue, I ordered that line again from the Zebrafish Repository Center. We got the line, but we needed to wait for 3 months for sexual maturation and to be able to differentiate females from males. We will now be able to finish with those experiments.

Regarding the noise exposure experiments. Our noise generating system broke down and the replacement parts were in backorder. We waited for 2 months to get the replacement and now the system is up and running.

6. Products

Part of the work was presented as a poster presentation at the Association for Research in Otolaryngology Midwinter Meeting held virtual during February 2022 and at the Molecular Biology of Hearing and Deafness, April 2022.

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7. Participants & Other Collaborating Organizations

Name:	*Xianghong Liu*
Project Role:	*Technician*
Nearest person month worked:	*9*
Contribution to Project:	*Mrs. Liu has performed experiments related to the derivatives screening. She is also in charge of the zebrafish husbandry and identification of the transgenic specimens.*
Funding Support:	*Mrs. Liu wage is complete supported by this award*

Name:	Lauren Barbush

Project Role:	*Master Student*
Nearest person month worked:	*6*
Contribution to Project:	*Ms. Barbush has been involved in all the experiments related to mice. She has been trained by the PI and she can proficiently perform ABRs and DPOAEs. She analyze the data, creates graphs, perform the statistical analysis and discuss with the PI the results. She also helps with mouse and fish husbandry.*
Funding Support:	*Ms. Barbush stipend is complete supported by this award*

8. Special Reporting Requirements

Other support

2R44DC018463-02A1 ZALLOCCHI (PI) 3.12 Cal Mo
Title: CDK2 inhibitors for protecting hearing loss
Supporting Agency: NIH/NIDCD
Name and address of the funding agency's procuring Contracting/Grants Officer: Doan, Hoai - National Institute on Deafness and Other Communication Disorders - 31 Center Drive, MSC 2320, Bethesda, MD USA 20892-2320
Performance period: 12/01/2021-11/30/2024
Description of the project goals: To determine TT001's therapeutic window in a mouse model for cisplatin ototoxicity and PK/PD studies in guinea pigs. This proposal will also address whether TT001 interferes with cisplatin's anticancer activity.
Specific Aims: To test how perform the necessary experiments to move TT001 forward into clinical trials to test its potential as an otoprotectant against cisplatin-induced hearing loss.
No overlap with existing proposal

2R44DC018762-02A1 ZALLOCCHI (PI) 3 Cal Mo
Title: Preclinical evaluation of an otoprotectant TT002
Supporting Agency: NIH/NIDCD
Name and address of the funding agency's procuring Contracting/Grants Officer: Doan, Hoai - National Institute on Deafness and Other Communication Disorders - 31 Center Drive, MSC 2320, Bethesda, MD USA 20892-2320
Performance period: 08/01/2022-07/31/2025
Description of the project goals: To determine TT002's therapeutic window in a mouse model for cisplatin ototoxicity and PK/PD studies in guinea pigs. This proposal will also address whether TT002 interferes with cisplatin's anticancer activity.
Specific Aims: To test how perform the necessary experiments to move TT002 forward into clinical trials to test its potential as an otoprotectant against cisplatin-induced hearing loss.
No overlap with existing proposal

9. Appendices

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