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14. ABSTRACT

In this study, we are examining the efficacy of a potential early intervention therapeutic for noise induced hearing loss (NIHL). The generation of reactive oxygen species is one of the underlying mechanisms of noise-induced damage to tissues in the inner ear that leads to noise-induced hearing loss (NIHL). The goal is to define an effective post-exposure, non-invasive intervention strategy to mitigate a primary cause of loud-sound induced hearing loss: mitochondrial dysfunction and overproduction of reactive oxygen species. Utilizing a small molecule mitochondrial fission inhibitor, mitochondrial division inhibitor-1, the results so far have demonstrated that inhibition of the mitochondrial fission process significantly attenuates NIHL and reduces damage to the tissues of the inner ear resulting from a single acute sound exposure.

15. SUBJECT TERMS
Noise induced hearing loss, mitochondria, reactive oxygen species, dynamin-related protein-1, mitochondrial division inhibitor-1

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1. INTRODUCTION

The generation of reactive oxygen species (ROS) is one of the underlying mechanisms of noise-induced damage to tissues in the inner ear that leads to noise-induced hearing loss (NIHL). Following loud sound exposure, the generation of excessive ROS by mitochondria in many different tissues of the inner ear is well recognized. Mitochondrial dysfunction, including the deregulation of fission and fusion processes, is implicated in many human pathological conditions including hearing loss. The studies proposed here will test the novel hypothesis that inhibiting the mitochondrial fission process will mitigate the deleterious effects of loud sound on hearing sensitivity. In our preliminary studies, we discovered that application of mitochondrial division inhibitor-1 (mdivi-1) to the outer ear canal after loud sound exposure significantly reduced noise-induced auditory threshold shifts in our mouse model of NIHL. Additionally, protection against outer hair cell loss at the high frequency responsive region of the organ of Corti was observed. Importantly, these findings demonstrated that altering mitochondrial dynamics following noise exposure is a potential mechanism for intervention of NIHL. In this study, through a pharmacological approach, we are defining a post-exposure intervention strategy to mitigate a primary cause of loud-sound induced hearing loss: mitochondrial dysfunction and overproduction of reactive oxygen species.

2. KEYWORDS

Hearing loss, loud sound, mitochondria, reactive oxygen species, dynamin-related protein-1, mitochondrial division inhibitor-1

3. ACCOMPLISHMENTS:

What were the major goals of the project?

A. Major Task 1: ACURO approval and equipment set-up. Dates: 09/15/2015 – 02/14/2016
B. Major Task 2: Identify effective dosing regimen for outer ear canal mdivi-1 application for a single loud sound exposure. Dates: 02/15/2016 – 08/14/2016
C. Major Task 3: Identify effective dosing regimen for intraperitoneal mdivi-1 injection. Dates: 08/15/2016 – 02/14/2017.
D. Major Task 4: Quantify cochlear mdivi-1 concentration. Dates: 02/15/2017 – 05/14/2017
E. Major Task 5: Identify molecular mechanisms affected by mdivi-1 treatment. Dates: 02/15/2017 – 03/14/2018.
F. Major Task 6: Identify effective dosing regimen for outer ear canal mdivi-1 application for multiple loud sound exposures. Dates: 02/15/2018 – 09/14/2018.

What was accomplished under these goals?

A. Major activities
   - Completion of Major Task 1: Sound exposure booth set-up and calibration.
   - Performance of studies within Major Task 2 through Major Task 6
   -

B. Specific objectives
   - Complete the set-up of the sound exposure equipment and isolation booth.
   - Completion of testing mdivi-1 efficacy in reducing noise induced hearing loss through application to the outer ear canal (Major Task 2).

- Initiation and completion of studies within Major Task 3, the identification of the most effective dosing regimen for intraperitoneal injection of mdivi-1.
- Performance of HPLC analysis of cochlear mdivi-1 concentrations in the inner ear (Major Task 4). This task is being completed with the assistance of the OHSU Bioanalytical Shared Resource/Pharmacokinetics Core.
- Completion of studies within Major Task 5, the identification of molecular mechanisms affected by mdivi-1 treatment.
- Initiation of studies within Major Task 6, identification of the most effective dosing regimen for outer ear canal mdivi-1 application for multiple loud sound exposures.

C. Significant results or key outcomes

Summary of major outcomes:
1. Major Task 2: Outer ear canal mdivi-1 application studies revealed that the 50μM immediate treatment protocol was most effective in reducing noise-induced threshold shifts.
2. Major Task 3: Intraperitoneal mdivi-1 injection studies revealed that 25 mg/kg administered immediately following noise exposure was the most effective dose for reducing noise-induced threshold shifts. Significant protection against OHC and synaptic ribbon loss was observed.
3. Major Task 4: HPLC analysis of plasma and cochlea samples showed that intraperitoneally injected mdivi-1was rapidly adsorbed into the bloodstream and crosses the blood-labyrinth-barrier.
4. Major Task 5: Mdivi-1 treatment reduced the noise-induced generation of cellular products of free radical species.
5. Major Task 6: Mdivi-1 treatment reduced loss of hearing sensitivity resulting from multiple sound exposures.

Major Task 1: ACURO approval and equipment set-up.

The isolation booth and sound exposure system was set-up and the calibration completed. This system is now in use for completion of the proposed work.

Major Task 2: Identify effective dosing regimen for outer ear canal mdivi-1 application for a single loud sound exposure.

We have completed this Task that involves outer ear canal application of mdivi-1 (50 μM and 150 μM) at either immediately following noise exposure or at 6 hours post-noise exposure. Further, based upon the results of these studies, we have also examined additional concentrations of mdivi-1 and performed a multiple-day (5 days) mdivi-1 dosing regimen.

Results:

1. *50 μM mdivi-1 immediate and 6 hours post-noise exposure treatment protocols*: Mdivi-1 (50 μM) was applied to one outer ear canal either immediately following sound exposure or at 6 hours post-noise exposure. The contralateral ear was treated with an equal volume of vehicle (0.23% DMSO). The results for the 50 μM immediate mdivi-1 treatment protocol, as measured by analysis of auditory brainstem response (ABR) and distortion product otoacoustic emissions (DPOAE) metrics, demonstrated significant protection against loss of hearing sensitivity at 2 weeks post-noise exposure (Figure 1). If treatment is delayed by

6 hours, a small protective effect against loss of hearing sensitivity was still observed. This does indicate that there is a "window of opportunity" following noise exposure for inhibiting mitochondrial fission and reducing noise-induced hearing loss. Histological analysis of OHC and synaptic ribbon counts following noise exposure showed that mdivi-1 treatment (50 µM) did not protect against OHC or synaptic loss (Figure 1).

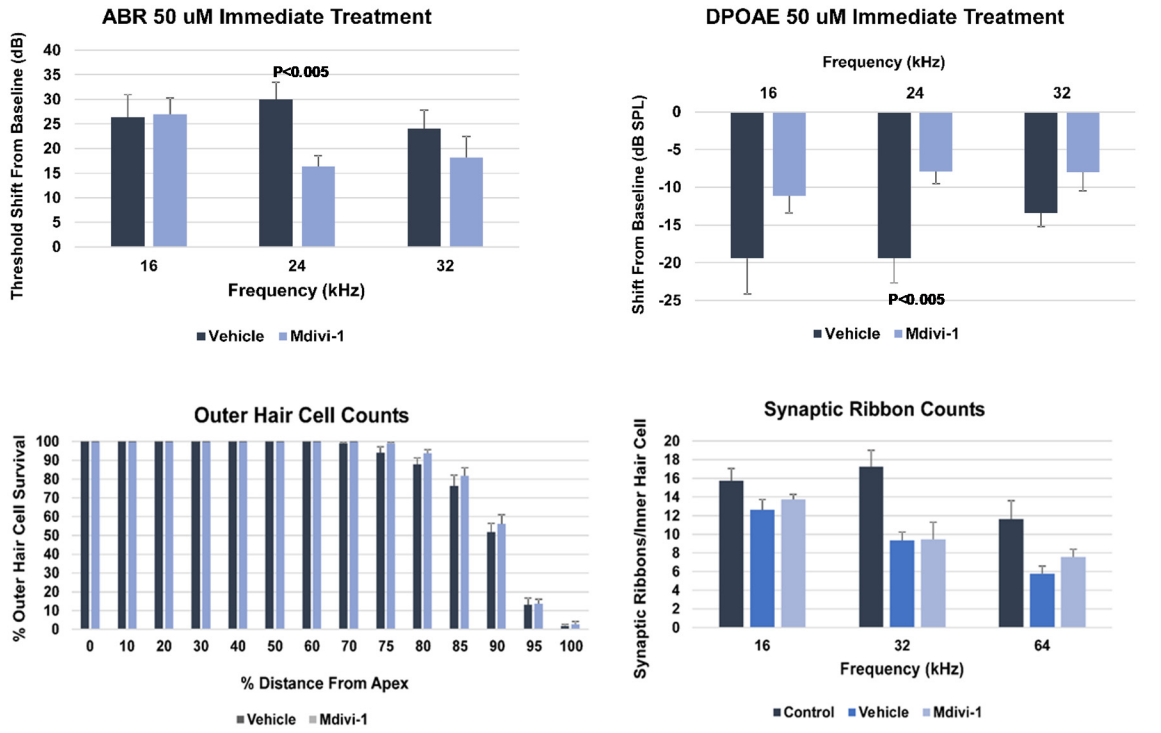


Figure 1. Low dose Mdivi-1 outer ear canal application immediately following loud sound exposure reduced loss of hearing sensitivity. Auditory brainstem response (ABR) and distortion product otoacoustic emission (DPOAE) metrics were measured prior to and again at 2 weeks post-noise exposure. Mice (n=16) were exposed to loud sound (103 dB SPL, 8-16 kHz) for 2 hours. Immediately following exposure, mdivi-1 (50 µM) was administered to one outer ear canal of each mouse. The contralateral ear received an equal volume of vehicle. Top panels: ABR analysis revealed a significant reduction in threshold shifts at 24 kHz. *P<0.005. Change of the cubic DPOAE (f1,f2=60 dB) with mdivi-1 treatment was also significant. Lower panels: OHC and synaptic ribbon counts performed after the final ABR/DPOAE measurements showed that this dose/route of mdivi-1 administration is not protective. Data are presented as mean+SEM.

2. *150 µM mdivi-1 immediate and 6 hours post-noise exposure treatment protocols*: The results demonstrated that this dose is not effective in reducing ABR threshold shifts following noise exposure. Twelve mice total were examined in this study group (12 cochlea each: vehicle + noise exposed and mdivi-1 + noise exposed). DPOAE analysis revealed a similar lack of significant effect with this higher dose. As discussed in our original grant application, the lack of a protective effect and even the induction of a harmful effect were potential

outcomes for the higher mdivi-1 dose. At high doses, mdivi-1 is capable of inhibiting protective mitophagy processes thereby allowing damaged/dysfunctional mitochondria to remain. For the results presented here, we believe that the most likely explanation for the lack of protection with the 150 μM mdivi-1 dose is the inhibition of mitophagy following noise exposure mitigating the protective effects of mdivi-1 on hearing sensitivity.

3. *Mdivi-1 dose response curve – immediate post-noise exposure treatment protocol*: To assess whether doses other than 50 or 150 μM would provide better protection against NIHL, we generated a dose-response curve for mdivi-1 treatment provided immediately following noise exposure. The doses included 25, 100, and 200 μM mdivi-1. We included the 200 μM mdivi-1 dose to determine whether a high dose would be detrimental to hearing sensitivity or to outer hair cell viability. The 25 and 100 μM doses were similar to the 50 μM dose in protection against ABR threshold shifts at 24 kHz. These different doses were studied in groups of 5 mice. The size of these groups were too small to allow for statistical significance (except for the 50 μM dose where n=16). Consistent with the 150 μM dose, the 200 μM dose was not effective in reducing noise-induced ABR threshold shifts relative to vehicle treated ears as measured at 2 weeks post-noise exposure (permanent threshold shifts).

Completion of outer hair cell counts on the 200 μM study group revealed that this higher dose did not reduce OHC survival in the treated ear relative to the vehicle treated ear following noise exposure. Importantly, these results indicate that, while not protective against ABR threshold shifts, a higher dose of mdivi-1 is not harmful to hearing sensitivity or OHC viability.

4. *Five-day mdivi-1 treatment protocol:* To determine whether multiple treatments would prove more protective, mdivi-1 (50 μM) was applied to one outer ear canal immediately following sound exposure and again at 24, 48, 72, and 96 hours post-noise exposure (n=5 mice). The contralateral ear was treated with an equal volume of vehicle (0.23% DMSO). Protection against permanent ABR threshold shifts at 24 kHz was indicated with the P value approaching statistical significance in this small group size (Figure 2). Importantly, the potential protection at 16 kHz is a new observation that was not achieved with a single mdivi-1 dose. DPOAE analysis also indicated that protection may be provided by the 5 day mdivi-1 treatment protocol. These findings are consistent with the knowledge that oxidative stress persists in the cochlea for up to 7 days following the sound exposure event

5. Statistical analysis of outer ear canal data: The OHSU Biostatistics and Design Program performed statistical analysis on the ABR and DPOAE data from the initial 6 mice in the single mdivi-1 treatment group and the initial 5 mice from the 5-day treatment regimen group. The key findings from the BDP's analysis is the following:

1. Single Mdivi-1 treatment (n=6 mice): The shift in ABR after 2 weeks was significantly ($F_{5,25} = 5.87$, $p = 0.001$) associated with treatment and frequency. Notably, at 2 weeks, the ABR threshold shift at 24 kHz was significantly lower in the mdivi-1 treated ear relative to vehicle (95% CI: 7.03-26.3 dB lower, p=0.002). For DPOAE, after accounting for frequency, the shift at 2weeks for the treated ear is estimated to be an average of 5.87 (95% CI: 0.28–11.45) units higher than for the untreated ear ($p = 0.04$).

2. Five day Mdivi-1 treatment group (n=5): Adjusted for frequency, the ABR shift for the treated ear was estimated to be 12.07 (95% CI: 4.52–19.6; p = 0.003) dB less than the ABR shift for the untreated ear. The statistician recommended repeating this study group in order to achieve higher statistical relevance.

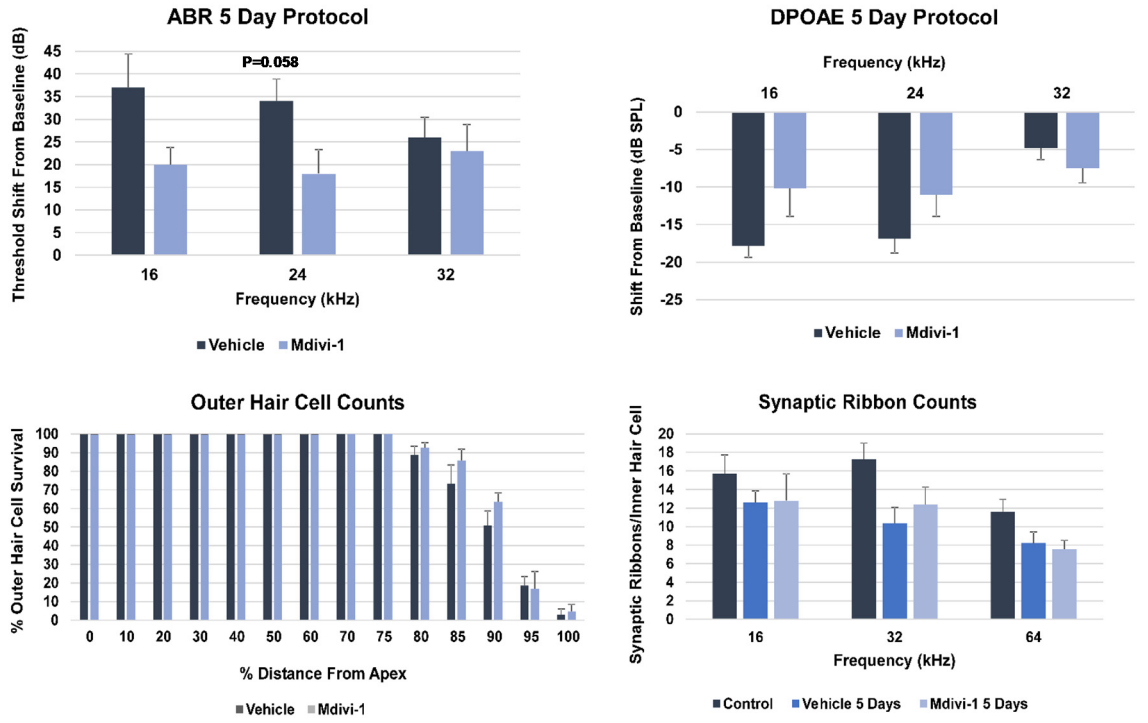


Figure 2. Five-day Mdivi-1 outer ear canal application reduced loss of hearing sensitivity. Auditory brainstem response (ABR) and distortion product otoacoustic emission (DPOAE) metrics were measured prior to and again at 2 weeks post-noise exposure. Mice (n=5) were exposed to loud sound (103 dB SPL, 8-16 kHz) for 2 hours. Immediately following exposure and again at 24, 48, 72, and 96 hours post-noise exposure, mdivi-1 (50 μM) was administered to one outer ear canal of each mouse. The contralateral ear received an equal volume of vehicle (0.23% DMSO). OHC and synaptic ribbon counts were performed after the final ABR/DPOAE measurements. Data are presented as mean+SEM.

Methods:

1. *Loud sound exposure*. The sound exposure level used in this study results in significant outer hair cell loss at the very high frequency region of the cochlea (>45 kHz) and no loss of inner hair cells. Mice are put into compartments of a divided wire mesh cage and placed into the center of an open field acoustic chamber. Free access to food and water is provided. For these single loud sound exposure studies, a free field noise level of 103 dB SPL, 8-16 kHz sound with a 5 minute ramp up in noise levels is applied for 2 hours. Control animals are kept at ambient noise levels for an equivalent amount of time.

2. *Outer ear canal application of mdivi-1*. Mdivi-1 is dissolved in DMSO to a stock solution of 100 mM and further diluted in saline immediately prior to use. For application of drug to the outer ear canal, the animal is lightly anesthetized with zylaxine (5 mg/kg)/ketamine (20

mg/kg) to prevent movement. Next, under a dissecting microscope, the animal is placed on their side with ear to be treated turned upwards, and 25 ul of the drug solution is applied to the outer ear canal using a pipette and sterile pipette tips. An equal volume of vehicle (saline + DMSO) is applied to the contralateral outer ear canal. The dissecting microscope allows proper placement of the pipette tip at the opening of the outer ear canal (at a safe distance from the tympanic membrane) and visual confirmation that the solution has gone into the ear canal and is up against the tympanic membrane without the formation of bubbles.

3. *Cochlear sensitivity measurements.* ABR (auditory brainstem response) threshold levels and DPOAE (distortion otoacoustic emissions) levels were measured before each experiment to confirm normal auditory function as well as to assess noise-induced hearing threshold shifts. The animals were anesthetized with a mixture of xylazine (10 mg/kg, IP) and ketamine (40 mg/kg, IP) and placed on a heating pad in a sound-isolated chamber. The external ear canal and tympanic membrane was inspected using an operating microscope to ensure the ear canal was free of wax and that there was no canal deformity, no inflammation of the tympanic membrane, and no effusion in the middle ear. For these studies, the individual performing the ABR/DPOAE measurements and analysis was blinded as to which ear of each animal had received the mdivi-1 versus saline treatment.

A. Auditory brainstem response: Needle electrodes are placed subcutaneously near the test ear, at the vertex and at the shoulder of the "test ear side." Each ear is stimulated separately with a closed tube sound delivery system sealed into the ear canal. The auditory brain-stem response to a 1-ms rise-time tone burst at 4, 8, 12, 16, 24, and 32 kHz is recorded and thresholds obtained for each ear. The intensity of tone burst stimulus is increased in steps of 5 dB. Threshold is defined as an evoked response of 0.2 μV from the electrodes.

B. Distortion product otoacoustic emissions: The "cubic" DPOAE at the frequency 2f1-f2 are generated by two tones played simultaneously to the ear. The stimuli consisted of two primary tones (f2/f1=1.2) at the level (L1=L2) 60 dB SPL that are emitted from speakers and presented over a range 4-32 kHz. The sound stimuli is generated by 24 bit 192 kHz ESI Wave terminal 192X Sound Card and an in house developed acoustic system. The DPOAE stimuli is delivered to the ear canal using a coupler tip fitted within the opening of the ear canal to form a closed acoustic system. The cubic distortion product is recorded in the ear canal by an Etymotic 10B microphone. The microphone is coupled to signal input channel of the sound card. For data analysis, the amplitude of the 2f1-f2 distortion product is plotted against the f2 frequency where a significant portion of the DP is generated.

4. *Auditory hair cell and synaptic ribbon counts*: Following ABR/DPOAE measurements, the mice were deeply anesthetized with ketamine hydrochloride (100 mg/kg) and 2% xylazine hydrochloride (10 mg/kg) and euthanized by cervical dislocation followed by decapitation. The cochleae were rapidly removed and perfused with 3.7% paraformaldehyde/0.25% glutaraldehyde in 0.1M phosphate buffer, fixed for 2.5 hours, and decalcified overnight in Tris/10% EDTA. The organ of Corti was dissected into five sections and incubated overnight with anti-CtBP2 antibody to label synaptic ribbons and anti-myosin 7A to label IHCs and OHCs. Following several PBS washes, the tissue sections were incubated with Alexa Fluor donkey anti-mouse 647 (CtBP2) and donkey anti-rabbit 568 (myosin 7A) secondary antibodies. The tissues were then stained with Alexa Fluor 488 phalloidin and Hoechst 33258 to allow for visualization and counting of outer hair cells. Confocal images were obtained on an Olympus IX81 inverted microscope fitted with an Olympus Fluoview FV1000 confocal laser microscope system. Cytocochleograms of each dissected cochlea were generated and the

number of hair cells present counted and plotted as fractional survival relative to percent distance from the cochlea apex following standard protocol. For synaptic ribbon counts, confocal z-stack projection were acquired at the 16, 32, and 64 kHz regions of the cochlea (identical regions of interest (ROI) were used consisting of 17 to 20 inner hair cells). The number of synaptic ribbons present per inner hair cell at each of the 3 frequencies was then counted. The individual performing the hair cell and synaptic ribbon counts was blinded as to which ear of each animal had received the mdivi-1 versus saline treatment.

Major Task 3: Identify effective dosing regimen for IP mdivi-1 application for a single loud sound exposure.

We have tested the efficacy of mdivi-1 intraperitoneal injections for attenuation of NIHL. This included 5, 15, and 25 mg/kg dosage administered immediately following noise exposure treatment.

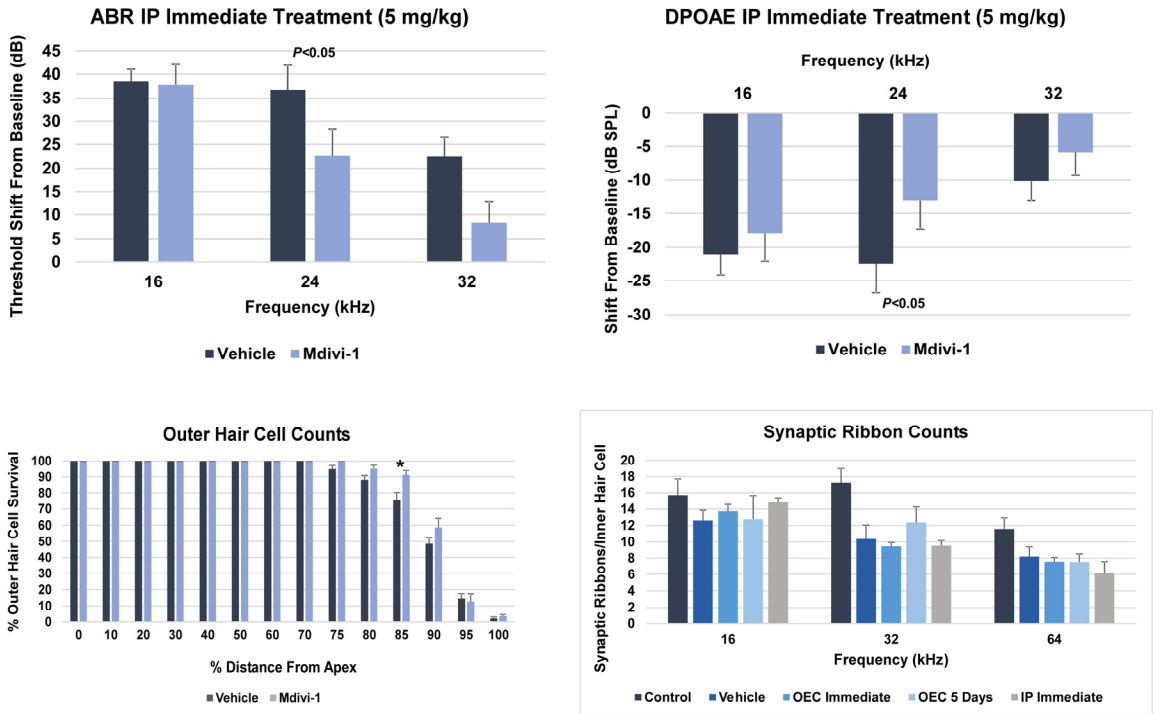


Figure 3. Low dose Mdivi-1 intraperitoneal injection immediately following loud sound exposure reduced loss of hearing sensitivity. Auditory brainstem response (ABR) and distortion product otoacoustic emission (DPOAE) metrics were measured prior to and again at 2 weeks post-noise exposure. Mice (n=10 vehicle/n=7 mdivi-1) were exposed to loud sound (103 dB SPL, 8-16 kHz) for 2 hours. Immediately following exposure, each mouse was IP injected with mdivi-1 (5 mg/kg) or vehicle (1.875% DMSO). Top panels: ABR analysis revealed a significant reduction in threshold shifts at 24 kHz. *P<0.05. Lower left panel: OHC counts performed after the final ABR/DPOAE measurements indicated that this dose/route of mdivi-1 administration may prove to be protective. Lower right panel: A comparison of synaptic ribbon counts from outer ear canal (OEC, 50 µM immediate), outer ear canal 5-day protocol (50 µM), and IP immediate injection (5 mg/kg) shows that mdivi-1 was not protective regardless of administration route. Data are presented as mean+SEM.

Results:

5 mg/kg mdivi-1 immediate post-noise exposure intraperitoneal injection treatment protocol: Mdivi-1 (5 mg/kg) or vehicle (1.875% DMSO) was IP injected immediately following loud sound exposure. The results for this treatment protocol, as measured by analysis of ABR and DPOAE metrics, demonstrated significant protection against loss of hearing sensitivity at 24 kHz ($P<0.05$) (Figure 3). Histological analysis of OHC at 2 weeks following noise exposure indicated that this treatment protocol may provide protection against hair cell loss with significance in protection being produced at 85% distance from the apex (at approximately the 50 kHz region of the basilar membrane). As with outer ear canal application of mdivi-1, 5 mg/kg intraperitoneal injection of mdivi-1 was not protective against synaptic ribbon loss.

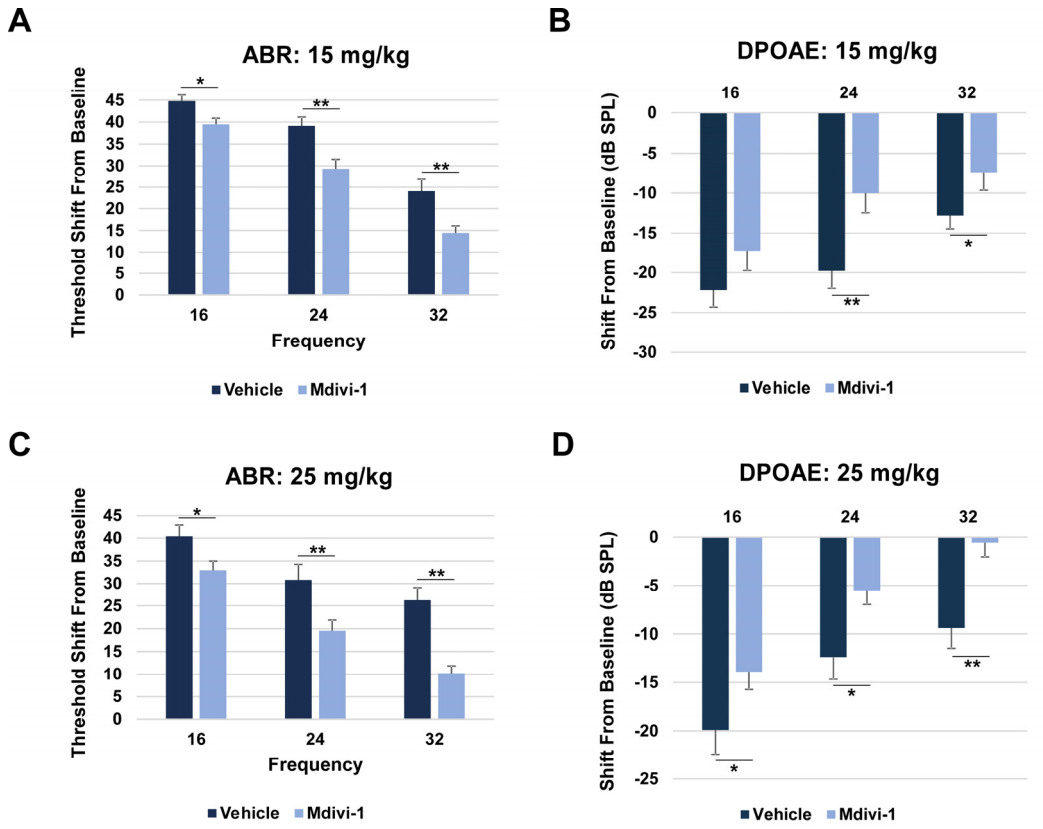


Figure 4. High dose Mdivi-1 single intraperitoneal injection immediately following loud sound exposure reduced loss of noise-induced hearing sensitivity. Auditory brainstem response (ABR) and distortion product otoacoustic emission (DPOAE) metrics were measured prior to and again at 2 weeks post-noise exposure. Mice were exposed to loud sound (103 dB SPL, 8-16 kHz) for 2 hours and immediately injected with either 15 mg/kg or 25 mg/kg mdivi-1 or vehicle. ABR threshold metrics were measured prior to and again at 2 weeks post-noise exposure. Numbers of animals tested were: 15 mg/kg mdivi-1 (n=18 mice); Vehicle for the 15 mg/kg dose (n=11); 25 mg/kg mdivi-1 (n=11); and Vehicle for the 25 mg/kg dose (n=12). * = $P<0.05$, ** = $P<0.01$

15 and 25 mg/kg mdivi-1 immediate post-noise exposure intraperitoneal injection treatment protocol: Due to the high aqueous insolubility of mdivi-1, we encountered difficulties in the administration of the higher doses (15 and 25 mg/kg) suspended in saline. Therefore, we administered mdivi-1 diluted in the widely used pharmacological agent, PEG 400. For the PEG 400 group, we diluted our mdivi-1 stock solution (125 mg/mL) in a 50% PEG 400/saline solution to achieve our desired final concentration. This formulation of mdivi-1 proved successful with the 15 and 25 mg/kg doses providing significant protection against hearing loss as determined by analysis of ABR and DPOAE metrics (Figure 4). Histological analysis of OHC at 2 weeks following noise exposure indicated that these higher mdivi-1 doses provided protection against noise induced damage to cochlear tissues (Figure 5). The OHC counts revealed that the 25 mg/kg mdivi-1 dose provided an approximately 20% increase in survival at the basal/high frequency region of the cochlea, corresponding to the 60 to 70 kHz tonotopic regions (Figure 5A). This effect was dose dependent as the 15 mg/kg dose provided less protection. Additionally, synaptic ribbon analysis demonstrated a small but statistically significant increase in the number of synaptic ribbons per IHC in the 25 mg/kg treatment group relative to vehicle (Figure 5B).

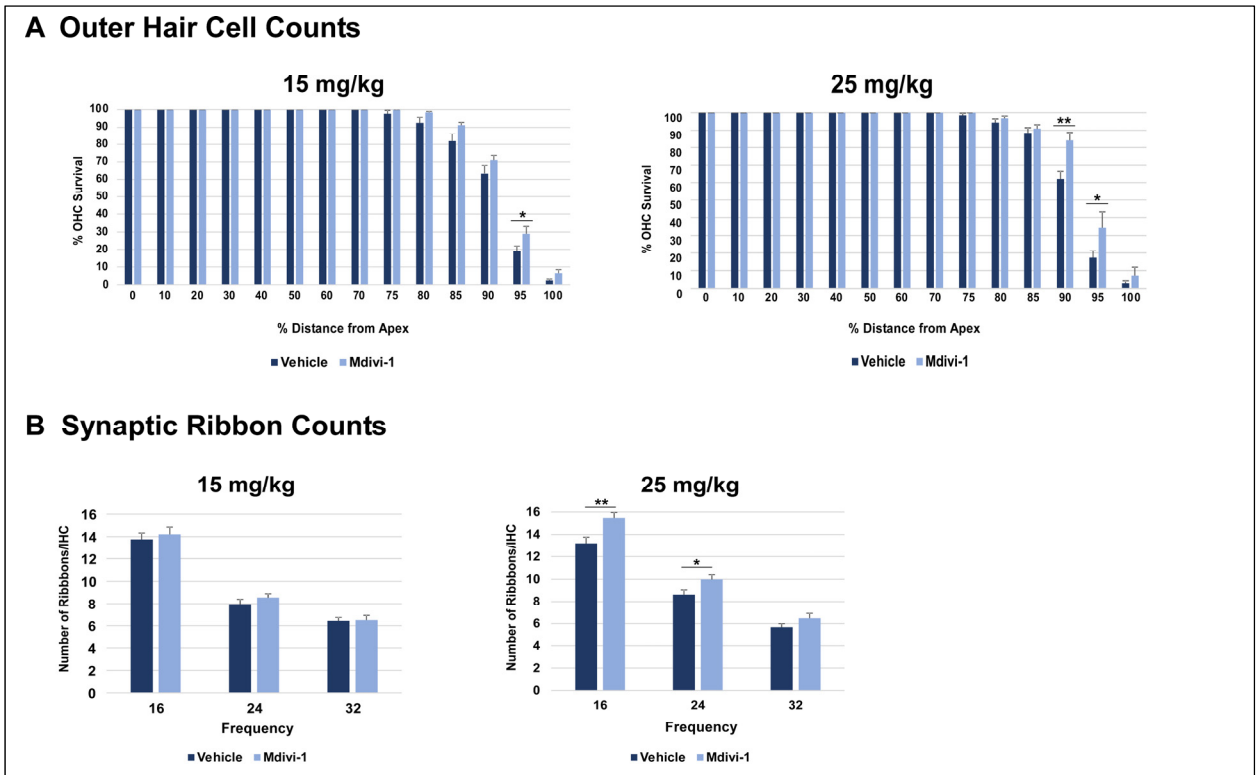


Figure 5. Mdivi-1 single intraperitoneal injection immediately following loud sound exposure reduced loss of OHCs and synaptic ribbons. Mice were exposed to loud sound (103 dB SPL, 8-16 kHz) for 2 hours and immediately injected with either 15 mg/kg or 25 mg/kg mdivi-1 or vehicle. Following the final ABR/DPOAE measurements at 2 weeks post-noise exposure for each group, the cochlea were harvested for morphological analysis. Numbers of animals analyzed were: 15 mg/kg mdivi-1 (n=15 mice); Vehicle for the 15 mg/kg dose (n=11); 25 mg/kg mdivi-1 (n=11); and Vehicle for the 25 mg/kg dose (n=12). * = $P<0.05$, ** = $P<0.01$.

The OHCs in the high frequency region of the cochlea are well known to be more susceptible to the pathological effects of loud sound. The basis for this is believed to be their higher (than those of the low frequency region) metabolic rate, a corresponding higher level of mitochondrial ROS production and lower levels of antioxidant enzymes. The hypothesis of this Major Task is supported by the combined reduction of noise-induced hearing threshold shifts and loss of OHCs and synaptic ribbons by inhibiting mitochondrial fission with mdivi-1. Thus, mdivi-1 as an early intervention treatment following noise exposure, is an effective treatment strategy for reducing damage to sensory epithelium and loss of hearing sensitivity. Importantly, this data provides a basis for considering that mdivi-1 may provide protection against hearing loss resulting from other and more damaging types of sound pressure levels including those generated by impulse and blast exposures.

Methods:

The methods for this Task were the same as described under Major Task 2.

Major Task 4: Quantify cochlear mdivi-1 concentration.

Mdivi-1 is a small lipophilic molecule that has previously been shown to cross the blood-brain barrier. However, as the blood-labyrinth barrier has been reported to be more restrictive to a variety of drugs and reagents, we performed studies to determine whether mdivi-1 crosses the blood-labyrinth barrier and to measure both the peak levels and the half-life of mdivi-1 in the cochlea and plasma.

Results:

The HPLC results demonstrated that mdivi-1 does cross the cochlear blood-labyrinth barrier and that mdivi-1 concentrations in the cochlea likely peak between 1 and 6 hours post-injection, persisting in low amounts out to 24 hours (Figure 6). HPLC analysis of plasma revealed a rapid adsorption of mdivi-1 into the bloodstream.

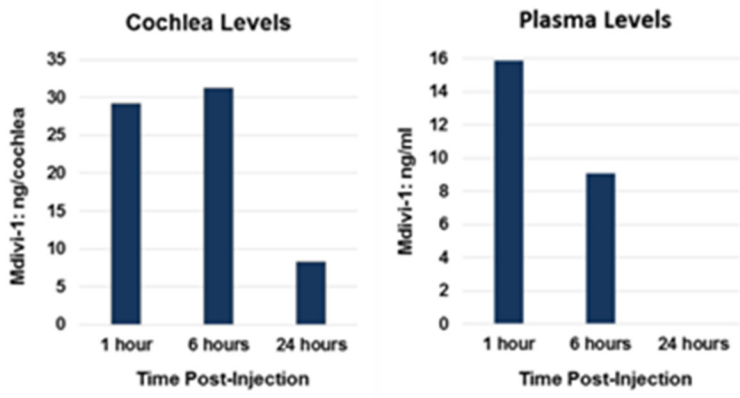


Figure 6. Mdivi-1 crosses the blood-labyrinth barrier. Mice were IP injected with 15 mg/kg mdivi-1 and the cochlea and plasma harvested over a time course post-injections. Mdivi-1 was not detected in plasma samples at 24 hours post-injection.

Methods: Mice were IP injected with 15 mg/kg mdivi-1 and the cochlea and plasma were harvested at the indicated time points post-injection. The cochlea were rapidly cleaned, in ice-cold PBS, of external tissue and blood and quick frozen in liquid nitrogen. For mdivi-1 extraction from cochlea, the cochlea from each mouse were homogenized with a pestle on ice and briefly sonicated. Plasma samples were generated by collecting blood in EDTA microtainer tubes. Mdivi-1 was extracted from the cochlea and plasma samples with acetonitrile. The supernatants were collected and HPLC analysis performed by the OHSU Biopharmacokinetics Core facility.

Major Task 5: Identify molecular mechanisms affected by mdivi-1 treatment.

The goal of this task is examine the cellular and molecular basis of mdivi-1's protective activity against the loss of hearing sensitivity. It is hoped that completion of these studies will further our understanding of the mechanisms of noise-induced damage to cochlear tissues and loss of hearing sensitivity leading to better future interventions and treatments for NIHL.

Results:

1. *Noise-Induced DRP1 Phosphorylation in Cochlear Tissues*: Phosphorylation of DRP1 results in its localization to mitochondria and induction of mitochondrial fission. Mdivi-1 inhibits the binding of DRP1 to mitochondria and subsequent fission processes, but not the

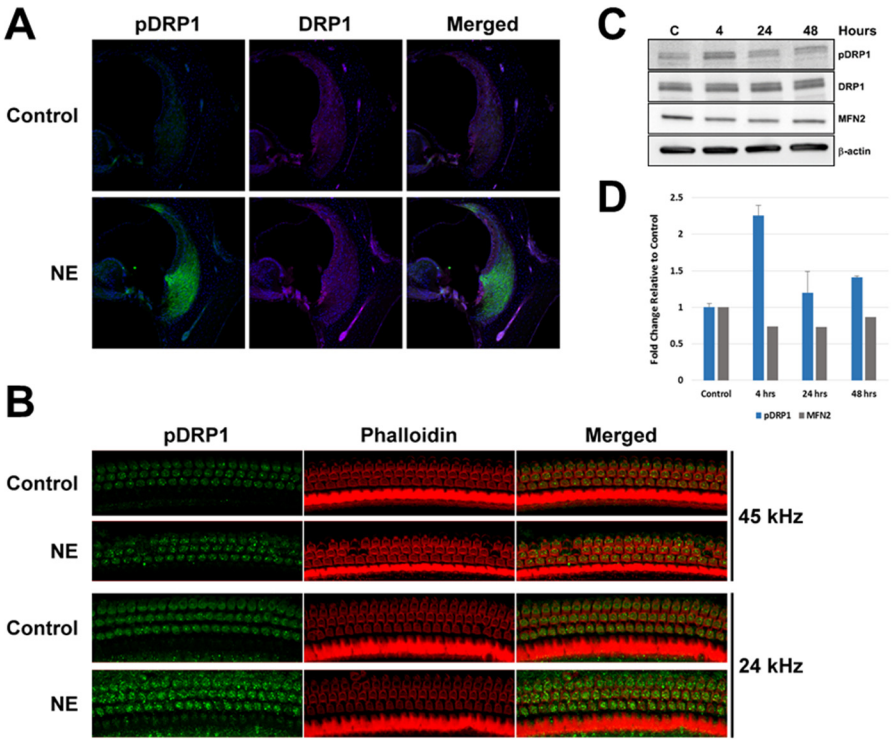


Figure 7. Loud sound exposure changes pDRP1 levels in the cochlea. **A.** Increased pDRP1 S616 fluorescent signal was observed in the cochlea lateral wall of sound exposed mice at 4 hours post-noise exposure relative to control (103 dB SPL, 8-16 kHz, 2 hours). **B**. Increased punctate pDRP1 S616 labeling was observed in OHCs at the 45 kHz region following noise exposure. **C**. Western blot analysis of whole cochlea extracts showed increased pDRP1 levels and decreased MFN2 levels at 4 hours post-noise exposure. **D.** Fold change of pDRP1 and MFN2 levels relative to control. Error bars = mean+SEM.

phosphorylation of DRP1. To examine the potential role of DRP1 in noise-induced damage to tissues of the cochlea, we assayed for the presence of phosphorylated pDRP1 in the murine cochlea following exposure to loud sound.

Loud sound exposure increased phosphorylation of DRP1 S616 in several areas of the cochlea including the type II fibrocytes of the lateral wall as well as in the OHCs (Figure 7). Interestingly, these different cell types are highly sensitive to noise-induced cell killing, and the sensitivity of both are thought to be based upon mitochondrial dysfunction. In OHCs, we observed increased pDRP1 levels at both the 24 kHz and 45 kHz regions. Additionally, the appearance phosphorylated DRP1 in the OHCs became highly punctate following noise exposure as would be expected if activated pDRP1 had translocated from the cytoplasm to the surface of mitochondria. For the 24 kHz region, while no OHC loss is observed at this region, mdivi-1 treatment provides significant protection against noise-induced loss of hearing at this frequency. Therefore, the prevention of mitochondrial dysfunction at this region provides a potential explanation for the protective effect of mdivi-1 against loss of hearing sensitivity at 24 kHz.

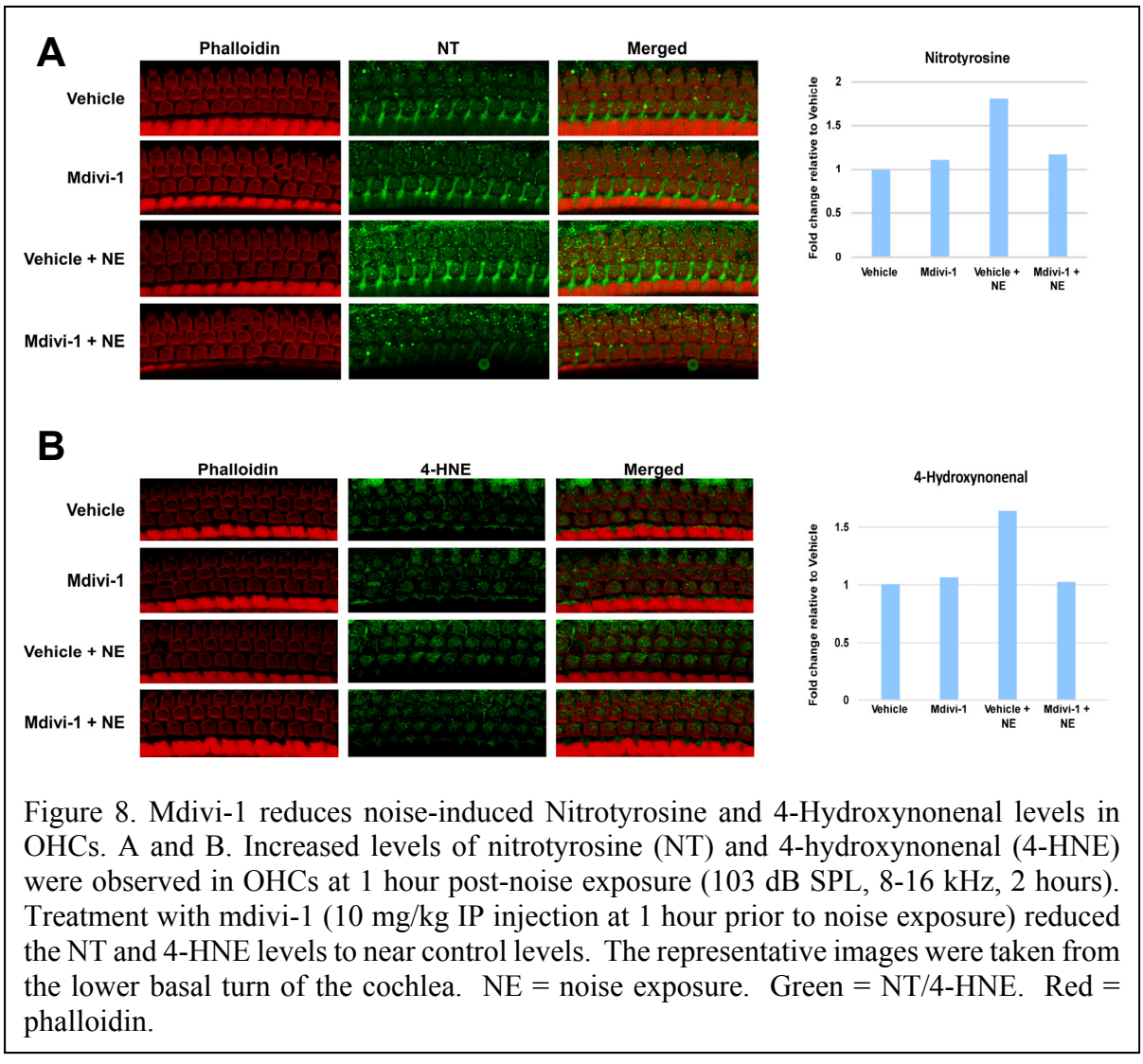


Figure 8. Mdivi-1 reduces noise-induced Nitrotyrosine and 4-Hydroxynonenal levels in OHCs. A and B. Increased levels of nitrotyrosine (NT) and 4-hydroxynonenal (4-HNE) were observed in OHCs at 1 hour post-noise exposure (103 dB SPL, 8-16 kHz, 2 hours). Treatment with mdivi-1 (10 mg/kg IP injection at 1 hour prior to noise exposure) reduced the NT and 4-HNE levels to near control levels. The representative images were taken from the lower basal turn of the cochlea. NE = noise exposure. Green = NT/4-HNE. Red = phalloidin.

2. *Attenuation of oxidative stress damage by Mdivi-1 treatment*: Here, we examined whether mdivi-1 could protect against the adverse mitochondrial-based cellular consequences of loud sound exposure by assessing the levels of 4-Hydroxynonenal (4-HNE), a marker of lipid peroxidation, and nitrotyrosine (NT), a biomarker of nitrogen free radical species, in OHCs following noise exposure (Figure 8). We observed that sound exposure increased the levels of both 4-HNE and NT in OHCs at 1 hour post-noise exposure. Treatment with mdivi-1 (10 mg/kg IP injection) at 1 hour prior to noise exposure inhibited the noise-induced increase of 4-HNE and NT levels in OHCs. This data supports our hypothesis that mdivi-1 can attenuate the deleterious loud sound-induced effects on mitochondria and the resulting cellular damage.

3. *Reduction of caspase-3/7 levels by Mdivi-1 treatment:* We asked whether mdivi-1 could reduce levels of OHC cell death by assaying for activation of caspases using the CellEvent Caspase-3/7 detection reagent (ThermoFisher) (Figure 9). We found that at 24 hours following loud sound exposure (103 dB SPL, 8-16 kHz, for 2 hours), a large number of fluorescent nuclei were present in the lower basal turn of vehicle treated (3% DMSO) mice (19/44). Treatment with mdivi-1 (25 mg/kg) 2 hours prior to sound exposure resulted in fewer positive nuclei (12/41). This data is consistent with the results of the outer hair cell counts for this dose of mdivi-1 (Figure 5). Images were acquired at the 50 kHz region of the cochlea. The 24-hour analysis time point was chosen as this is an early point in the programmed death process.

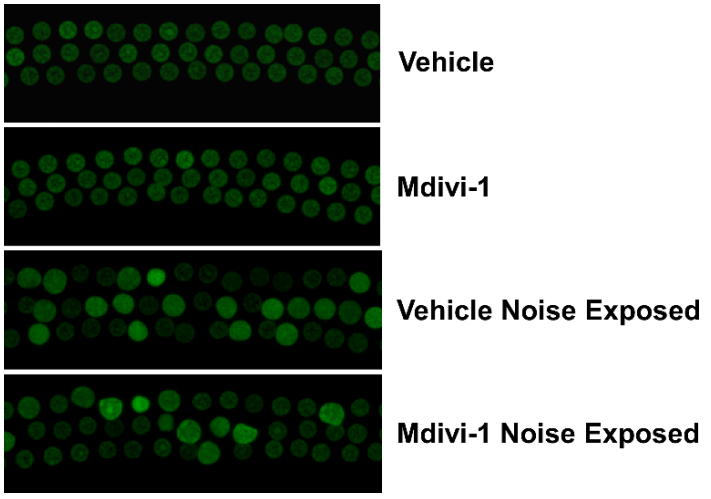


Figure 9. Mdivi-1 reduces noise-induced activated caspase-3/7 levels in OHCs. Increased levels of activated caspase-3/7 were observed in OHCs at 24 hours post-noise exposure (103 dB SPL, 8-16 kHz, 2 hours). Treatment with mdivi-1 (25 mg/kg IP injection at 2 hours prior to noise exposure) reduced the number of positive nuclei relative to vehicle treated. The representative z-stack projection images were taken from the 50 kHz location in the lower basal turn of the cochlea. Green = activated capase-3/7.

Methods:

Immunological analysis of pDRP1, 4-HNE, and NT levels in OHCs: Mice were IP injected with mdivi-1 (10 mg/kg) or vehicle (3.75% DMSO) 1 hour prior to exposure to loud sound (103 dB SPL, 8-16 kHz for 2 hours) or ambient noise levels. At the indicated times post-sound exposure, the mice were deeply anesthetized with ketamine hydrochloride (100 mg/kg) and 2% xylazine hydrochloride (10 mg/kg) and euthanized by cervical dislocation followed by decapitation. The cochleae were rapidly removed and perfused with ice cold 3.7% paraformaldehyde/0.1% glutaraldehyde in 0.1M phosphate buffer and fixed overnight at 4^0C.

Following overnight decalcification in Tris/10% EDTA, each organ of Corti was dissected into five sections and incubated overnight with anti-4-HNE, anti-NT, or anti-pDRP1 S616 antibody. Following several PBS washes, the tissue sections were incubated with Alexa Fluor 488 (pDRP1) or 568 (4-HNE and NT) secondary antibodies. Phalloidin 647 and Hoechst 33258 staining allowed for visualization of outer hair cells. Confocal images were obtained on an Olympus IX81 inverted microscope fitted with an Olympus Fluoview FV1000 confocal laser microscope system. Cytocochleograms of each dissected cochlea were generated. Confocal z-stack projection images were acquired at the specified tonotopic regions of the cochlea (identical regions of interest (ROI) were compared for each different test group and consisted of 17 to 20 inner hair cells each).

Immunological analysis of activated caspase-3/7 levels in OHCs: Mice were IP injected with mdivi-1 (25 mg/kg) or vehicle (3% DMSO) 2 hours prior to exposure to loud sound (103 dB SPL, 8-16 kHz for 2 hours) or ambient noise levels. At 24 hours post-sound exposure, the mice were deeply anesthetized with ketamine hydrochloride (100 mg/kg) and 2% xylazine hydrochloride (10 mg/kg) and euthanized by cervical dislocation followed by decapitation. The cochleae were rapidly removed and perfused with CellEvent Caspase-3/7 detection reagent and incubated at 37^0C for 30 minutes. The cochlea were then fixed in 4% paraformaldehyde for 3 hours followed by overnight decalcification in Tris/10% EDTA. Next, each organ of Corti was dissected into five sections and stained with phalloidin 647 and Hoechst 33258. Confocal z-stack projection images were acquired at the 50 kHz region of the cochlea (identical regions of interest (ROI) were compared for each different test group and consisted of 17 to 20 inner hair cells each).

Major Task 6: Identify effective dosing regimen for outer ear canal mdivi-1 application for multiple loud sound exposures.

Multiple temporary threshold shift (TTS)-only inducing loud sound exposures have been found to result in cumulative synaptic ribbon loss and permanent loss of hearing sensitivity after three exposure events (significant elevation of ABR thresholds, approximately 20 dB from baseline at 24 and 32 kHz). Our proposed studies examined whether outer ear canal application of mdivi-1 (50 μM), provided immediately following multiple (TTS)-inducing sound exposures (100 dB SPL, 2 hours, 8-16 kHz), would provide protection against loss of hearing sensitivity.

Results: In this study, mdivi-1 (50 μM) was applied to one outer ear canal of each mouse immediately following loud sound exposure (100 dB SPL, 8-16 kHz for 2 hours) (n=7 mice). Vehicle was applied to the contralateral ear canal. ABR and DPOAE measurements were taken two weeks later. The cycle was repeated one week later for a total of 5 sound exposures. The results demonstrated a much smaller permanent threshold shift than expected after 5 loud sound exposures (Figure 10A) with no difference between the two treatment groups. As expected from previous studies, there was no reduction in DPOAEs. Determining whether inhibiting mitochondrial fission can reduce the cumulative effects of multiple noise exposures on hearing function over time is a highly important goal for this study. Therefore, we continued our studies utilizing a slightly higher sound level in order to produce more cumulative damage while still keeping each individual exposure within the TTS-inducing range (101 dB SPL, 8-16 kHz for 2 hours). The mice were exposed to the same cycle of noise exposure and ABR/DPOAE as before. This time, mdivi-1 was administered as an IP injection of 25 mg/kg,

a dose that provided protection against loss of hearing sensitivity and increased OHC survival (Figures 4 and 5). After 4 sound exposures, we observed significant protection against loss of hearing sensitivity at 24 kHz (n=3 mdivi-1, n=3 vehicle).

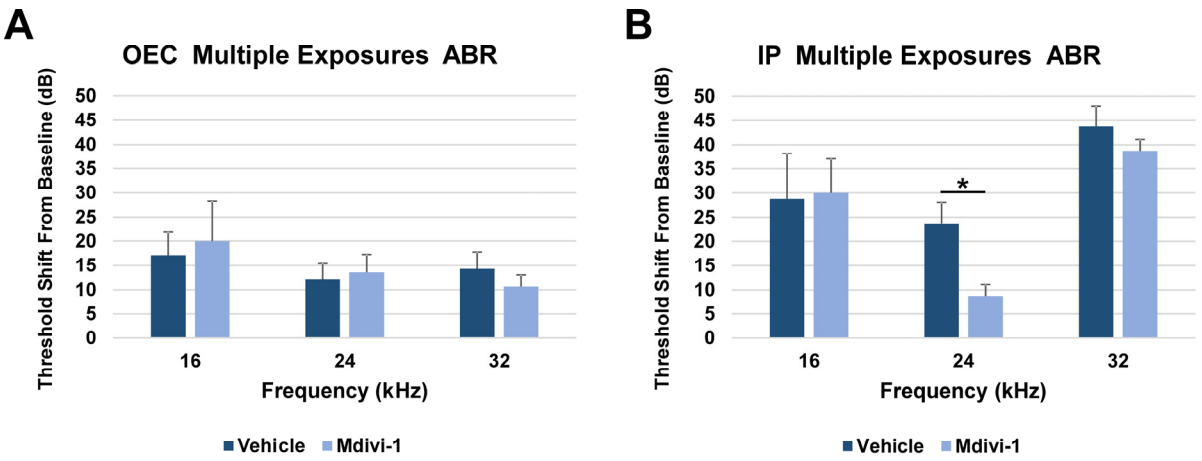


Figure 10. Efficacy of mdivi-1 in reducing multiple sound exposure induced threshold shifts. A. Mice were exposed to loud sound (100 dB SPL, 8-16 kHz) for 2 hours and immediately treated with 50 µM mdivi-1 in one outer ear canal. ABR/DPOAE metrics were measured 2 weeks later. This cycle was repeated 5 times (n=7 mice). B. Mice were exposed to loud sound (101 dB SPL, 8-16 kHz) for 2 hours and immediately injected (IP) with 25 mg/kg mdivi-1. ABR/DPOAE metrics were measured 2 weeks later. This cycle has been repeated 4 times to date (n=3 mdivi-1, n=3 vehicle treated mice). Error bars = mean+SEM.

D. Other achievements

None to report.

What opportunities for training and professional development has the project provided?
Nothing to report

How were the results disseminated to communities of interest?
Nothing to report

What do you plan to do during the next reporting period to accomplish the goals?

We will continue to work toward completion of the goals of Major Task 6 and will complete the study by the end of the no-cost extension period.

4. IMPACT

What was the impact on the development of the principal discipline(s) of the project?

In this project, we are examining the efficacy of a potential early intervention therapeutic for noise induced hearing loss (NIHL). The generation of reactive oxygen species (ROS) by mitochondria is an underlying mechanism of noise-induced damage to tissues in the inner ear that leads to noise-induced loss of hearing sensitivity. Our results demonstrate that inhibition of the

mitochondrial fission process with a small molecule inhibitor significantly attenuates NIHL and reduces damage to the tissues of the inner ear. Additionally, this study is utilizing a localized application of the therapeutic molecule to the outer ear canal that limits systemic exposure and allows for a higher local concentrations in the cochlea. The observed reduction in noise-induced loss of hearing sensitivity and increased auditory hair cell survival with both outer ear canal application and intraperitoneal injection of mdivi-1 demonstrates the efficacy of this drug as a therapeutic agent for NIHL. This study also introduced and substantiated the therapeutic potential of directly targeting mitochondrial dynamics and dysfunction for protection against NIHL. This is in contrast to previous studies on potential NIHL protective agents that have primarily focused on the use of antioxidants and vitamin supplements.

What was the impact on other disciplines?
Nothing to report

What was the impact on technology transfer?
Nothing to report

What was the impact on society beyond science and technology?
Nothing to report

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change
Nothing to report

Actual or anticipated problems or delays and actions or plans to resolve them
Due to the delays in the delivery of the sound exposure booth, we continue to be behind the dates of the approved SOW schedule. We have made every attempt to catch up with the SOW and will complete Major Task 6 in this no-cost extension period.

Changes that had a significant impact on expenditures
Nothing to report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Nothing to report
Significant changes in use or care of human subjects
Nothing to report

Significant changes in use or care of vertebrate animals
Nothing to report

Significant changes in use of biohazards and/or select agents
Nothing to report

6. PRODUCTS
Publications, conference papers, and presentations

Nothing to report

Journal publications
Nothing to report

Books or other non-periodical, one-time publications
Nothing to report

Other publications, conference papers, and presentations
1. ARO (2017), Baltimore, MD: Inhibition of DRP1 Activity Reduces Noise Induced Hearing Loss. Poster.

Website(s) or other Internet site(s)
Nothing to report

Technologies or techniques
Nothing to report

Inventions, patent applications, and/or licenses
Nothing to report

Other Products
Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

1. Name: Alfred Nuttall
   Project Role: PI
   Nearest Person Month Worked: 2 Calendar months
   Contribution to Project: Dr. Nuttall assisted in data analysis to ensure that the experiments and the noise exposure, ABR, and DPOAE systems continue to progress and function correctly.

2. Name: Teresa Wilson
   Project Role: Co-I
   Nearest Person Month Worked: 9 Calendar months
   Contribution to Project: Dr. Wilson worked on the experimental projects of Major Tasks 1 through 6.

3. Name: Edward Porsov
   Project Role: Engineer
   Nearest Person Month Worked: 1 Calendar months
   Contribution to Project: Mr. Porsov routinely calibrated and monitored the noise exposure, ABR, and DPOAE systems to ensure that they continue to function correctly.

4. Name: Sarah Foster

Project Role: Research Assistant
Nearest Person Month Worked: 12 Calendar months
Contribution to Project: Ms. Foster worked on the experimental projects of Major Task 1 through 6.

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

What other organizations were involved as partners?
Nothing to report

8. SPECIAL REPORTING REQUIREMENTS

QUAD CHARTS: An updated Quad chart is attached to this report

Controlling Mitochondrial Dynamics to Mitigate Noise-Induced Hearing Loss

MR141227
W81XWH-1-0560

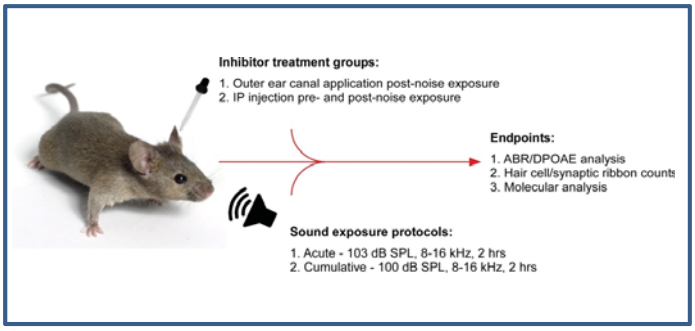
PI: Nuttall, Alfred L. **Org:** Oregon Health & Science University **Award Amount:** $1,233,279

Study Aims

• *Specific Aim 1*: Determine the optimal dose and dosing regimen for outer ear canal application and intraperitoneal injection of mdivi-1 for mitigation of NIHL resulting from a single steady-state noise exposure.
•*Specific Aim 2*: Determine whether inhibition of mitochondrial fission can protect against the adverse mitochondrial-based cellular consequences of loud sound exposure.
• *Specific Aim 3*: Determine whether outer ear canal administration of mdivi-1 will prevent the cumulative effects of multiple steady-state loud sound exposures over an extended time period.

Approach

The well-characterized CBA/CaJ mouse model will be used to determine the optimal dose and timing of inhibitor administration in the prevention of NIHL. Functional tests for hearing sensitivity will include auditory brainstem responses and distortion product otoacoustic emissions measurements. Molecular and cellular endpoints will be examined for the mitochondrial-dependent mechanisms leading to tissue damage, and the inhibitor's ability to attenuate these resulting in reduced NIHL.



Accomplishment: Task 3 (IP administration) has been completed and Task 6 is in progress (multiple sound exposure/immediate mdivi-1 treatments).

Timeline and Cost

Activities CY	15	16	17	18
Regulatory approvals and equipment ordering/set-up				
Specific Aim 1: Single steady-state induced NIHL, mdivi-1 dose and timing optimization				
Specific Aim 2: Etiology of mdivi-1 protection against NIHL				
Specific Aim 3: Cumulative steady-state induced NIHL, dose and timing optimization				
Estimated Budget ($K)		**$479**	**$371**	**$383**

Updated: 10/13/2018

Goals/Milestones

CY15 Goals – Obtain regulatory approvals and equipment ordering
- ■ ACURO approval
- ■ Equipment ordering and set-up: ABR/DPOAE and sound exposure systems have been installed.

CY16 Goal – Mdivi-1 protection against acute loud sound exposure
- ■ Outer ear canal inhibitor application: Task 2 is in progress.
- ■ IP injection of inhibitor: single immediate treatment is nearly completed
- ◘ HPLC quantification of cochlea inhibitor concentration is in progress

CY17 Goals – Molecular and cellular basis of mdivi-1 protection
- ■ Examine markers of mitochondrial health
- ◘ Quantify mitochondrial ROS production following noise exposure

CY18 Goal – Mdivi-1 protection against multiple loud sound exposures
- ❑ Outer ear canal inhibitor application after multiple sound exposures

Comments/Challenges/Issues/Concerns

We continue to be behind the SOW schedule, but are making up significant time.

Budget Expenditure to Date

Projected Expenditure: $1,233,279 (Total costs)
Actual Expenditure: $1,094,941 (Total costs).