Cisplatin-Induced Ototoxicity and the Effects of Intratympanic Diltiazem in a Mouse Model

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Sponsorships or competing interests that may be relevant to content are disclosed at the end of this article.

Abstract

Objective. To evaluate whether the calcium-channel blocker diltiazem has protective effects against cisplatin-induced ototoxicity in a mouse model.

Study Design. Original basic science in vivo investigation.


Subjects. Thirty-nine female CBA/J mice.

Methods. Pure tone- or click-evoked auditory brainstem responses (ABRs) were recorded in CBA/J mice to determine auditory thresholds. All mice had baseline ABRs recorded. They were then given a single cisplatin bolus (14 mg/kg), followed by 5 consecutive days of intratympanic diltiazem or saline control. Follow-up thresholds were recorded on days 7, 14, and 21 postcisplatin. Tone-evoked ABRs evaluated the otoprotective effect of 2-mg/kg diltiazem in 9 mice, and dose effect was examined in response to click-evoked ABR with 2- or 4-mg/kg diltiazem in 2 groups of 15 mice.

Results. Saline-treated ears had significantly elevated tone-evoked auditory thresholds when compared with diltiazem-treated ears (P = .038) on day 7 postcisplatin only. Click-evoked ABR thresholds were significantly elevated in saline-treated ears versus diltiazem-treated ears for the 2-mg/kg group (P = .001) and 4-mg/kg group (P = .011) on days 7, 14, and 21 postcisplatin.

Conclusion. Intratympanic diltiazem has significant protective effects against cisplatin ototoxicity at 2 and 4 mg/kg.

Keywords

ototoxicity, calcium, cisplatin, diltiazem, hearing loss

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Cisplatin is a chemotherapeutic agent used in the treatment of many solid tumors, including head and neck cancers. One of the dose-limiting side effects of cisplatin is ototoxicity, which manifests as tinnitus and/or bilateral sensorineural hearing loss in the clinical setting.¹⁻⁴ Ototoxicity caused by cisplatin has effects on a number of inner ear structures, including the stria vascularis, supporting cells, spiral ganglion cells, and outer hair cells (OHCs).²⁻⁵⁻⁶ However, the OHCs appear to be the most susceptible to damage. There is evidence that the mechanism of ototoxicity is related to formation of reactive oxygen species that damage OHCs of the cochlea and trigger apoptosis.⁵⁻⁷ Research has explored otoprotective compounds that work by interfering with this reactive oxygen species pathway,⁸⁻⁹ yet otoprotective agents have not translated to clinical practice.

An important contributor to this mechanism of apoptosis-induced ototoxicity is calcium. The relationship of calcium homeostasis in regulating a variety of responses in hearing has recently been discussed.⁵⁻¹⁰ Apoptosis is a dynamic event that requires coordination of many enzymatic events that ultimately lead to activation of degradative enzymes. A number of enzymes are activated during apoptosis, such as caspases⁶ and calpains,¹¹ which are calcium-activated proteases. Thus, a major contributor to apoptosis is calcium influx.¹²⁻¹³ In vitro experiments have shown that reactive oxygen species induce calcium influx to the OHCs of the guinea pig cochlea likely through voltage-sensitive calcium channels.¹⁴ Specifically, L-type calcium channels appear to be responsible for calcium influx leading to apoptosis.¹⁵ In vivo studies have suggested that diltiazem, an L-type calcium channel blocker (CCB), may reduce the concentration of calcium precipitates at the basolateral membrane of the OHCs.¹⁶ There is also evidence demonstrating that L-type calcium channels can be localized to the inner ear and play

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a role in the pathogenesis of acoustic injury. The use of L-type calcium channel blockers has been experimented with to protect against acoustic trauma.\textsuperscript{17}

Despite the knowledge of the role of calcium in apoptosis and auditory physiology, there has been little exploration of inhibiting calcium influx as a potential therapy for cisplatin-induced hearing loss. The few studies having explored CCBs against cisplatin used in vitro models. Notably, So et al used an organ of Corti–derived cell line to show that flunarizine, a T-type CCB, demonstrated a protective effect against cisplatin ototoxicity.\textsuperscript{18} Liang et al proposed that calcium-dependent potassium channels are responsible for cisplatin-induced apoptosis in spiral ligament cells; thus, by blocking calcium channels, it prevents potassium-related events causing injury.\textsuperscript{19}

We previously determined the dose of cisplatin that induces otoxicity with an acceptable mortality rate in a murine model and demonstrated partial protective effects of intratympanic (IT) dexamethasone.\textsuperscript{20} Pharmacologic studies have shown that the concentration of steroid compounds administered IT is elevated in the inner ear as compared with other routes of administration.\textsuperscript{21} Other pharmacokinetic studies have shown that compounds are absorbed through the round window membrane and that drug delivery to the inner ear is due to passive diffusion of the compounds.\textsuperscript{22} IT injections appear to be an efficient method of drug delivery that is easily translatable to clinical practice for the otolaryngologist. It avoids the potential complications of systemic side effects, and it prevents systemic metabolism of the drug before it takes effect.

With this background knowledge, we hypothesized that IT administration of the L-type CCB diltiazem would be protective against cisplatin-induced ototoxicity in a mouse model.

Methods
This study was approved by the Institutional Animal Care and Use Committee at the University of Connecticut Health Center (100191-0214, 100943-0917).

Animal Subjects
This study was performed in 2 phases. Eighteen ears of 9 female 4-week-old CBA/J mice from Jackson Laboratories (Bar Harbor, Maine) were used for phase 1 of this study. Sixty ears of 30 female 4-week-old CBA/J mice were used in phase 2. The mice were weighed each day before any procedure. Prior to cisplatin injections, they were kept in standard housing. Following administration of cisplatin, they were housed in chemotherapy-treated animal isolation. They had free access to food and water. Each day that procedures took place, they were observed closely for any signs of weight loss, isolation, poor social interaction, and general distress.

Study Design

Phase 1. Otoprotective effects of IT diltiazem were evaluated. Baseline tone-evoked auditory brainstem responses (ABRs) were recorded in 9 mice (18 ears). Cisplatin (14 mg/kg) was injected on day 1. On the same day, IT treatment was initiated wherein 9 ears were treated with 5 consecutive days of 2-mg/kg diltiazem, while the contralateral ears were treated with 5 consecutive days of saline as a control. Follow-up ABRs were recorded on postcisplatin days 7, 14, and 21.

Phase 2. Otoprotective effects of 2 doses of IT diltiazem were evaluated in this phase. The design in this phase was similar to phase 1, except that ABRs were click evoked and contralateral ears did not serve as saline controls; rather, separate sets of control animals were utilized. A total of 30 mice were used. In the 2-mg/kg experiment, 15 mice (30 ears) received either IT diltiazem (14 ears) or saline (16 ears). In the 4-mg/kg experiment, 15 mice (30 ears) received either IT diltiazem (16 ears) or saline (14 ears).

Auditory Brainstem Responses
All ABR testing was performed while the mice were under inhalational general anesthesia in a sound-proof chamber. The ear not being tested was obstructed with putty and a cotton ball to minimize any crossover-evoked responses. The ABRs were measured with a system from Tucker-Davis Technologies (Alachua, Florida). A speaker was placed at the opening of the external canal of the mouse. Subcutaneous electrodes were placed with the active electrode at the vertex, the ground electrode behind the right ear, and the reference electrode behind the left ear.

During phase 1, ABRs were recorded to assess thresholds to pure tone bursts presented to 1 ear. Five-millisecond pure tone bursts of 8, 16, 24, and 32 kHz were delivered at a rate of 21/s. The stimuli were presented at intervals of 5 dB, ranging from 20 to 90 dB of sound pressure level. The 10-ms recorded signal was amplified 100,000 times and filtered between 30 and 3000 Hz. The signals were averaged after 512 presentations. In phase 2, ABRs were recorded in response to broadband clicks. Alternating polarity clicks of 100 microseconds were presented 500 times and averaged. The stimulus was attenuated in 5-dB steps until no response was present. ABR threshold was defined as highest level that evoked ABR peak III.

Injections/Anesthesia
Prior to any IT injection, general anesthesia was administered. Induction anesthesia of 3.5% to 4% isoflurane was administered with concomitant 0.8 to 1.0 L of oxygen in a closed chamber. After induction, the mouse was transferred to a snout-masked anesthesia device where maintenance anesthesia was administered at 2.0% to 2.5% with 0.4 to 0.8 L of oxygen.

Intraperitoneal injections with cisplatin, 14 mg/kg, were performed with a 27-gauge needle on a tuberculin syringe, followed by a 1-mL bolus of saline to ensure hydration.

While the mouse was on the masked maintenance anesthesia, an operating Zeiss otomicroscope (Dublin, California) and otic speculum were used to view the tympanic membrane.
Once the TM was visualized, a 30-gauge needle connected to a microsyringe via Silastic tubing was placed into the middle ear through the TM. A minimum of 5 mL of either diltiazem in 0.9% normal saline or 0.9% normal saline was delivered into the middle ear until it was visualized filling the middle ear space. After IT injection, the mouse was left under anesthesia for at least 3 minutes to facilitate diffusion into the inner ear. A small amount of solution was occasionally seen on the external auditory canal, which was insignificant when compared with the volume of solution in the middle ear.

A dose of diltiazem in adults with hypertension can be up to 120 mg. In a 70-kg male, this is roughly 2 mg/kg. Thus, in phase 1, a 2-mg/kg diltiazem solution in 0.9% normal saline was prepared for IT injections. This dose was calculated by estimating the weight of the mice to be on average 25 g and by estimating about a 5-mL IT injection (10-mg/mL solution).

In phase 2, two solutions of diltiazem were prepared for IT injections: a 2-mg/kg dose (10-mg/mL solution) and a 4-mg/kg dose (20 mg/mL) based on an average weight of 25 g per mouse and a 5-mL IT injection.

**Statistical Analysis**

The Berndtson method was used to determine that a minimum of 8 replicates were needed to detect a 10% difference from control with a coefficient of variability of 5% for 95% power at \( P < .05 \). In phases 1 and 2, ABR thresholds differences were analyzed with repeated measures analyses of variance (ANOVAs; drug \( \times \) day \( \times \) stimulus frequency, dose \( \times \) day, or drug \( \times \) day). \( P \leq .05 \) was considered statistically significant. To simplify graphic representation of data, percentage change in ABR thresholds from baseline (day 0) was calculated for display purposes.

**Results**

Phase 1 tone-evoked ABR results through day 21 postcisplatin for 2-mg/kg diltiazem- and saline-treated ears are shown in Figure 1, respectively. In all figures, error bars represent standard deviation. ABR thresholds were elevated in both groups, with the largest magnitude of change being on days 7 and 14. There was a trend toward recovery of thresholds in both groups by day 21, although thresholds remained above baseline. Magnitude of ABR threshold change appeared similar between the groups, except at day 7, when diltiazem-treated ears had relatively little shift in thresholds. Three-way repeated measures ANOVA revealed the main effect of day narrowly missing statistical significance (\( P = .051 \)) and no influence of drug. Closer analysis of the day variable was performed on ABRs comparing diltiazem- and saline-treated ears as a function of day. This analysis showed that day 7 ABR recordings in ears treated with diltiazem had a significantly lower threshold shift as compared with ears treated with saline (\( P = .038 \)), independent of stimulus frequency. This is graphically represented as a percentage change from baseline for saline and diltiazem (Figure 2).

While these data demonstrate promising beneficial effect for IT diltiazem, the effect was small. Several modifications were introduced in phase 2. First, the number of ears was increased from 9 to 30. In addition, because of a lack of a stimulus frequency effect, we replaced pure tone bursts with clicks. We avoided a possible effect of diltiazem on the contralateral, saline-treated ear by using a separate group of control animals in phase 2. Finally, in addition to a 2-mg/kg diltiazem group, a 4-mg/kg group was added to evaluate a dose effect.

With these changes, there was a significant difference of ABR thresholds between the saline and diltiazem groups. Two-way repeated measures ANOVAs showed significantly lower click-evoked ABR thresholds at both 2-mg/kg (\( P = .001 \)) and 4-mg/kg (\( P = .011 \)) diltiazem when compared with each corresponding saline control group. This effect was not diminished as a function of day. ANOVA comparing the difference in thresholds of the 2- and 4-mg/kg experiments showed no significant differences, suggesting that a higher dose did not provide additional protection. Figure 3 demonstrates this as a percentage change from...
baseline in ABR thresholds comparing the average of all saline-treated mice with the average of 2- and 4-mg/kg diltiazem mice. While large threshold elevations were seen in the saline-treated groups, ABR thresholds in diltiazem-treated groups had little change from baseline. The trend toward improved threshold on day 21 seen in phase 1 was also seen in phase 2. Nevertheless, thresholds remained about 30% above baseline.

**Discussion**

Cisplatin has dose-limiting ototoxic side effects that manifest as tinnitus and bilateral sensorineural hearing loss. Mechanisms of cisplatin-induced ototoxicity have been well established, and the role of calcium is paramount in this process.4,6,10 We hypothesized that preventing calcium influx through targeted inhibition of OHC L-type calcium channels will provide otoprotection from the apoptosis-induced effects of cisplatin. Our results are consistent with this hypothesis, and diltiazem-treated ears showed statistically significant reduction in ABR threshold versus control ears.

Our findings extend in vitro findings of CCB otoprotective properties against cisplatin4,14,18,19 to in vivo findings. L- and T-type calcium channels have been shown to exist in the inner ear,4,14,17-19 and diltiazem has been shown to reduce calcium precipitate concentration in OHC by blocking calcium channels at the basolateral membranes of the OHCs.16 Otoprotective properties of CCB appear to extend beyond ototoxic agents. A recent study demonstrated that calcium-blocking anticonvulsants can be protective against noise-induced hearing loss.23 Together these results provide strong support for use of CCBs as otoprotective agents against ototoxicity. In this study, diltiazem was used because it is an antagonist of L-type calcium channels, which provided a targeted approach to prevent calcium-mediated apoptosis of cisplatin ototoxicity. Diltiazem was chosen for its L-type calcium channel specificity, but other CCBs may be beneficial. It would be of interest to evaluate the additive otoprotective effects of both L- and T-type CCBs against cisplatin in future experiments.

In phases 1 and 2, it appeared that maximal cisplatin threshold shifts occur around day 14 after cisplatin treatment. We administered IT diltiazem for the first 5 days after cisplatin injection. Potentially longer duration of IT treatment could increase the beneficial effects. Of course, pretreatment with diltiazem may have additional benefits that could be explored.

There was a trend for ABR threshold shift toward baseline in the saline-treated ears with incomplete recovery, while the diltiazem-treated ears had consistent levels of otoprotection. Recovery of ABR thresholds have been demonstrated in mice, and electrophysiological recovery has been reported in guinea pigs.25,26 Using the single-dose cisplatin model, we previously showed that maximum ABR threshold elevations occurred at day 14 with slight recovery by day 21, with stability of elevated thresholds thereafter.27 We also demonstrated similar results using a multidose cisplatin model with stable and elevated thresholds up to day 90.25 These changes in threshold are not unique to mouse models of cisplatin ototoxicity. Stengs et al demonstrated electrophysiologic recovery in guinea pigs with compound action potentials and cochlear microphonics.26 Therefore, in our study, day 21 was chosen as the final time point, because ABR threshold changes have stabilized at 3-week postadministration of cisplatin.

One in vitro study found that the effect of CCBs against cisplatin was dose dependent.18 We tested 2 doses of diltiazem in phase 2 of the study and demonstrated continued otoprotective effects for both doses against cisplatin ototoxicity. The 2- and 4-mg/kg IT diltiazem groups showed statistically significant protection throughout the 21-day follow-up period. Both doses afford significant protection; thus, 2 mg/kg appears to offer an adequate dose for protection. Further studies can be performed to evaluate whether there is dose dependency at lower doses of diltiazem.

In our study, click- and tone-evoked ABRs were used to evaluate the effects of diltiazem as an otoprotective agent. We and others previously demonstrated that cisplatin causes significant click- and tone-evoked ABR threshold shifts.20,28 In phase 1, there was no statistically significant effect of

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Figure 2. Phase 1. Mean percentage change in auditory brainstem response thresholds on day 7 after cisplatin injection for saline- and diltiazem-treated ears (data from Figure 1).

Figure 3. Phase 2. Mean percentage change in click-evoked auditory brainstem response thresholds of intratympanic saline and 2 and 4 mg/kg of diltiazem on days 7, 14, and 21 after injection of cisplatin.
stimulus frequency in tone-evoked ABRs; thus, click-evoked ABRs were used for phase 2. Both represent valid audiometric evaluations for cisplatin ototoxicity models, and other animal studies have used click-evoked ABRs to evaluate otoprotection.28,29

IT injections provide a route of drug delivery that is easily translatable into clinical practice for the otolaryngologist. IT therapy has been shown to provide adequate pharmacokinetic uptake into the inner ear, without unwanted side effects.21,22 A small amount of systemic uptake from IT injections is likely. Blood pressure and heart rate were not measured after IT injections of diltiazem in our mice; however, the mice were observed closely for signs of distress after each procedure. Nevertheless, in a clinical setting, heart rate and blood pressure should be evaluated if IT diltiazem was to be utilized.

We have found that IT injection of a compound in one ear can have a measurable effect on the other ear. For example, we previously demonstrated that IT injections of vancomycin had increased ABR threshold in the contralateral saline control ear.30 Studies have also demonstrated the presence of gentamicin in the contralateral ear following IT injections, and the cochlear aqueduct appears to be the potential site of transfer to the contralateral ear.31 In phase 2, we continued with IT injections of diltiazem as opposed to systemic administration because it offered more direct, consistent uptake to the inner ear and lowered the needed dose to minimize systemic side effects. In addition, we did not inject the contralateral ear with the saline control. This may account for a larger difference in auditory thresholds between the diltiazem and saline ears in phase 2 versus phase 1. Further pharmacokinetic and cerebrospinal fluid studies following IT administration of otoactive agents need be performed to evaluate mechanisms of contralateral effects.

Based on pharmacologic studies, the concentration of IT drug delivery is highest at the basal turn of the cochlea, and for a drug to reach the apical turns, repeated or continuous injection may be necessary.22 This makes IT injections even more appealing as a therapeutic option since cisplatin tends to affect high-frequency basal-turn OHCs. Histologic examination of hair cells exposed to cisplatin with and without diltiazem would also be a useful next step in evaluating otoprotection from cisplatin at a cellular level. Finally, as drug delivery methods to the inner ear continue to evolve, we feel that diltiazem should be a strong consideration as an agent that serves as a potential otoprotectant against inner ear damage.

Here we demonstrated that IT diltiazem had significant otoprotective effects against cisplatin-induced ototoxicity at 2 and 4 mg/kg. This study is the first to suggest diltiazem as a potentially new otoprotective agent against the ototoxic effects of cisplatin.

Author Contributions
James G. Naples, conception hypothesis and design of work; acquisition, analysis, and interpretation of data; drafting and approval of manuscript; presentation of work; Kourosh Parham, design of work; analysis and interpretation of data; drafting and approval of manuscript.

Disclosures
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