

Effect of coenzyme A on outer hair cells in cisplatin ototoxicity: functional and ultrastructural study

Francisco Fernández-Cervilla^{1,3}, María Martínez-Martínez¹, Eduardo Fernández-Segura^{2,3},
Francisco Javier Cañizares-García^{2,3} and Pascual Vicente Crespo-Ferrer²

¹Department of Otorhinolaryngology, ²Department of Histology, School of Medicine and ³Institute of Neuroscience, Biomedical Research Centre, University of Granada, Granada, Spain

Summary. The aim of this study was to use functional and morphological analyses to evaluate the protective effect of coenzyme A (CoA) in cisplatin-induced toxicity in outer hair cells (OHC). Three groups of 8 guinea pigs were used: control (group I), cisplatin-treated (group II) and cisplatin + CoA-treated (group III). In groups II and III, a single ototoxic dose of cisplatin (10 mg/kg) was injected intraperitoneally. Group III was co-treated with CoA (900 µg/kg per day for 7 consecutive days). Electrocochleography (ECoG) recordings were made before and after the 7-day treatment period in all groups. After ECoG on day 7, all animals were anesthetized and the cochleae were removed and fixed for ultrastructural analysis. Cell damage in OHC was observed with transmission electron microscopy. Cisplatin induced a significant increase in auditory thresholds ($p < 0.001$) compared to group I (control). In contrast, group III (cisplatin + CoA) had significantly reduced thresholds ($p < 0.001$) compared to the group treated with cisplatin alone (group II). We found no significant differences between the control group and animals co-treated with cisplatin and CoA. The electron microscopy findings in OHC were consistent with these results. Ultrastructural analysis of OHC in group II showed morphological indications of necrosis, i.e. cytoplasmic swelling and vacuolation, and mitochondrial swelling. In group III the cell morphology of OHC was preserved, with ultra-

structural characteristics similar to the control group. In conclusion, co-treatment with cisplatin with CoA inhibited antineoplastic-induced cytotoxicity in OHC in a guinea pig model.

Key words: Cisplatin, Coenzyme A, Outer hair cells, Ototoxicity, Otoprotection

Introduction

Cisplatin is a chemotherapeutic agent used in the treatment of several types of cancer. Its therapeutic efficacy, however, is limited by the appearance of adverse effects such as peripheral neuropathy, kidney failure and ototoxicity (van Ruijven et al., 2004). The ototoxic effects of cisplatin have been studied in humans (Yancey et al., 2012) and experimental animals (Franceschi et al., 2011). Some studies found that guinea pigs treated with cisplatin had hearing loss at high frequencies, as determined by electrocochleography (ECoG) (Fernandez-Cervilla et al., 1993), auditory brainstem-evoked potentials and otoacoustic emissions (Sockalingam et al., 2000). In addition, cisplatin was found to increase auditory thresholds and latencies, and to decrease the amplitudes of some waves (van Ruijven et al., 2005).

These functional alterations are related with cellular injury in the basal coil of the cochlea, which can spread posteriorly to the remaining coils (van Ruijven et al., 2005). Morphological studies have shown that cellular injury induced by cisplatin appears mainly in the supporting cells and outer hair cells (OHC) (van Ruijven

Offprint requests to: Francisco Fernández-Cervilla MD, PhD; Departamento de Otorrinolaringología, Facultad de Medicina, Universidad de Granada, E-18071 Granada, España. e-mail: franfer@ugr.es

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et al., 2004), whereas alterations in the inner hair cells are minimal or absent (Ciges et al., 1996). Injury may also appear in the organ of Corti, stria vascularis and spiral ganglion (Tabuchi et al., 2011).

Several compounds have been used to diminish the ototoxic effects induced by cisplatin, such as thiosulfate (Berglin et al., 2011). Also, recent studies have shown that resveratrol (Erdem et al., 2012) and dexamethasone (Waissbluth et al., 2013) are effective in reducing these undesired effects of cisplatin. Previous work by our group with functional, ECoG and scanning electron microscopic (SEM) methods found that coenzyme A (CoA) and pantothenic acid had a protective effect in guinea pigs treated with cisplatin (Ciges et al., 1996).

In the present study we investigated the toxic effects of cisplatin on OHC with ECoG and transmission electron microscopy (TEM). In addition, we tested the protective effect of CoA on cisplatin-induced cytotoxicity in these cells of the cochlea.

Materials and methods

Animals and drugs

We used three groups of 8 albino guinea pigs (24 animals in all) weighing 200-300 g. In the control group (group I), no treatment was used. In the cisplatin group (group II) the animals were treated with cisplatin. In the cisplatin + CoA group (group III) the animals were treated with cisplatin and CoA simultaneously. All animals were handled in accordance with current Spanish legislation (Real Decreto 52/2013) and European Communities Council Directives (2010/63/EU). The experimental protocol was approved by the University of Granada Research Ethics Committee.

The drugs used were cisplatin (Neoplatin, Bristol-Myers SA, Madrid, Spain) and coenzyme A (Aluzime[®], Laboratorios Alter SA, Madrid). Both drugs were dissolved in distilled water to a concentration of 1

mg/ml. Cisplatin was administered intraperitoneally in a single dose (10 mg/kg). We selected a single high dose of cisplatin (near the LD50) to ensure inner ear damage in a model of acute toxicity in guinea pigs (Fernandez-Cervilla et al., 1993; Watanabe et al., 2003). Coenzyme A was also administered intraperitoneally at a daily dose of 900 μ g/kg for 7 consecutive days. Diuresis was not induced, and all animals had free access to water and food.

Electrocochleography

ECoG results were recorded with an evoked potential audiometer (Amplaid MK6, Amplaid, Irvine, CA, USA) by implanting a permanent electrode in the round window in each animal as previous reported (Ciges et al., 1996). Briefly, the animals were anesthetized for this procedure with ketamine 120 mg/kg, atropine 0.4 mg/kg and chlorpromazine (Largactil) 4 mg/kg. The electrodes transmitted compound action potentials, which we used to determine auditory thresholds and latencies to the appearance of the N1 wave (ms) at each signal intensity. Recordings were started at 120 dB sound pressure level (SPL) and the signal was decreased in 10-dB steps until the wave of interest was no longer detected. Auditory stimulation consisted of unfiltered clicks with a duration of 100 ms, a stimulation rate of 10/s and at least 500 stimuli at a sensitivity of 5 μ V.

In groups II and III, ECoG recordings were made before the drugs were given and after 7 days. In group I (control) recordings were obtained at the start and at the end of the treatment period.

Table 1. Measurement of compound action potential latencies (N1 wave) in control, cisplatin-treated and cisplatin-CoA co-treated guinea pigs ^{a,b}.

	Control	Cisplatin	Cisplatin + CoA
120 dB SPL	1.699±0.471	2.296±0.692	1.391±0.283
110	1.811±0.484	2.531±0.711	1.431±0.165
100	1.941±0.492	2.722±0.712	1.602±0.164
90	2.201±0.579	2.908±0.900	1.800±0.184
80	2.311±0.591		1.901±0.198
70	2.432±0.594		2.050±0.132
60	2.360±0.876		2.340±0.367

^a Auditory stimulation consisted of unfiltered clicks lasting 100 ms, a stimulation rate of 10/s and at least 500 stimuli at a sensitivity of 5 μ V.

^b Data are presented as the mean \pm SD.

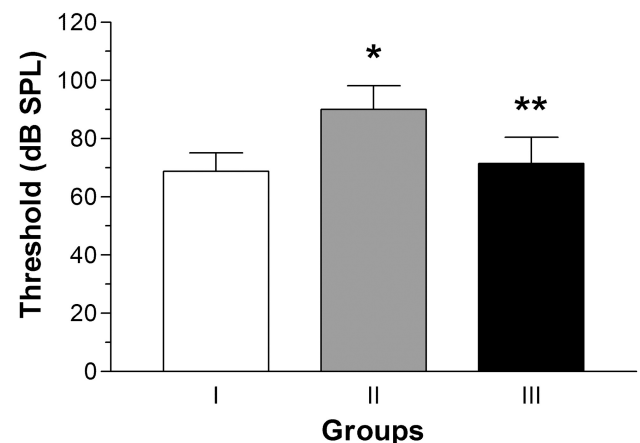


Fig. 1. Auditory thresholds from control, cisplatin-treated and cisplatin + CoA co-treated guinea pigs. Data are presented as the mean \pm SD obtained from 8 animals. Statistically significant differences between control and treated groups are indicated with an asterisk (* $p < 0.001$, comparison between control and cisplatin-treated groups; ** $p < 0.001$, comparison between cisplatin and cisplatin + CoA treated groups).

*CoA and cisplatin in OHC**Transmission electron microscopy*

After ECoG recordings were obtained, all animals were anesthetized and decapitated, and the organ of Corti was removed and immediately fixed in 2.5% glutaraldehyde with phosphate buffer at pH 7.4 and 4°C

for 6 hours. For postfixation we used 2% buffered osmium tetroxide with potassium ferrocyanide for 1 hour at 4°C. Block staining was done with 2% uranyl acetate in aqueous solution for 1 hour at 4°C in the dark. Ultrathin sections (50 nm) were prepared and mounted on 300-mesh copper grids, and contrasted with 2%

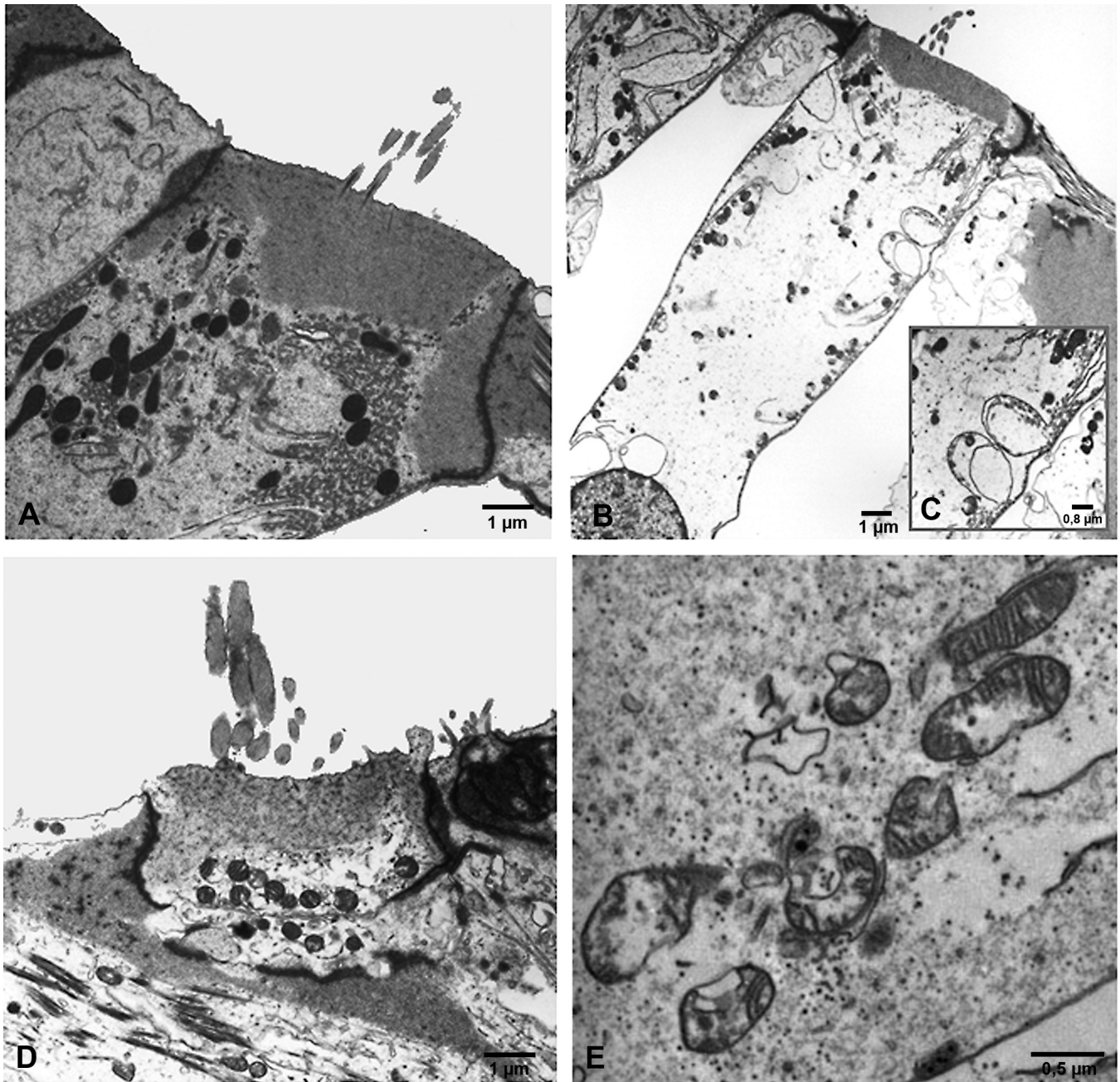


Fig. 2. Cellular ultrastructure of outer hair cells (OHC) in control and cisplatin-treated groups examined with transmission electron microscopy. **A.** Control, normal OHC characterized by intact cellular organelles and plasma membrane. **B-E.** OHC from guinea pigs treated with cisplatin (10 mg/kg) characterized by intracellular swelling and areas of degeneration (**B**), intracytoplasmic vacuolization (**C**) and atypical mitochondria with vacuolation and swelling (**E**). The cilia were unaltered and appeared normal (**D**).

uranyl acetate in distilled water for 10 min followed by lead citrate for 5 min (Renau and Megías, 1998). Ultrastructural observations were made with a Zeiss 902 TEM (Zeiss, Oberkochen, Germany).

Statistical analysis

Data were expressed as the mean \pm standard deviation (SD). Differences between the mean thresholds in the experimental groups were analyzed with one-way analysis of variance (ANOVA) followed by the Bonferroni multiple comparisons test. Differences between means were considered statistically significant when $p < 0.05$. All statistical analyses were done with the GraphPad In-Stat 3.00 program for Windows (GraphPad Software, San Diego, CA, USA).

Results

Electrocochleography

Table 1 shows ECoG results of latencies of N1 wave in each of the three groups. In the cisplatin-treated group (group II) the latencies N1 wave was delayed and the threshold was increased. In the cisplatin + CoA group (group III) all thresholds and latencies were normal.

Quantitative data for the auditory threshold in each group are shown in Fig. 1. In the control group (group I) the auditory threshold appeared at 68.75 ± 6.40 dB SPL. In the cisplatin group (group II) this threshold was detected at 90 ± 8.16 dB SPL. However, in the cisplatin + CoA group (group III) the auditory threshold appeared at 71.42 ± 8.99 dB SPL. Treatment with cisplatin thus induced a significant increase in auditory thresholds ($p < 0.001$) compared to the control group. Also, the reduced auditory threshold in group III (cisplatin + CoA) compared to group II (cisplatin) led to significant differences ($p < 0.001$) between these groups. In contrast, there were no significant differences between the cisplatin + CoA group (group III) and the control group (group I).

Transmission electron microscopy

Fig. 2 shows the ultrastructural features of OHC in control guinea pigs and animals treated with cisplatin. In the cisplatin group, OHC were characterized by cytoplasmic swelling, loss of the normal pattern of organization of the smooth endoplasmic reticulum beneath the plasma membrane, and the presence of intracytoplasmic and perinuclear vacuoles (Fig. 2A-C). Transmission electron microscopic images showed no alteration in the patterns of organization of the cilia (Fig. 2D). However, the mitochondria were characterized by vacuolation and swelling with remnants of cristae (Fig. 2E). No alterations were seen in the nucleus. In the cisplatin + CoA group, the ultrastructural appearance of the OHC was similar to the control group. The smooth endoplasmic reticulum had a normal pattern of

organization, and no alterations were seen in the mitochondria (Fig. 3).

Discussion

We used ECoG and ultrastructural analysis to

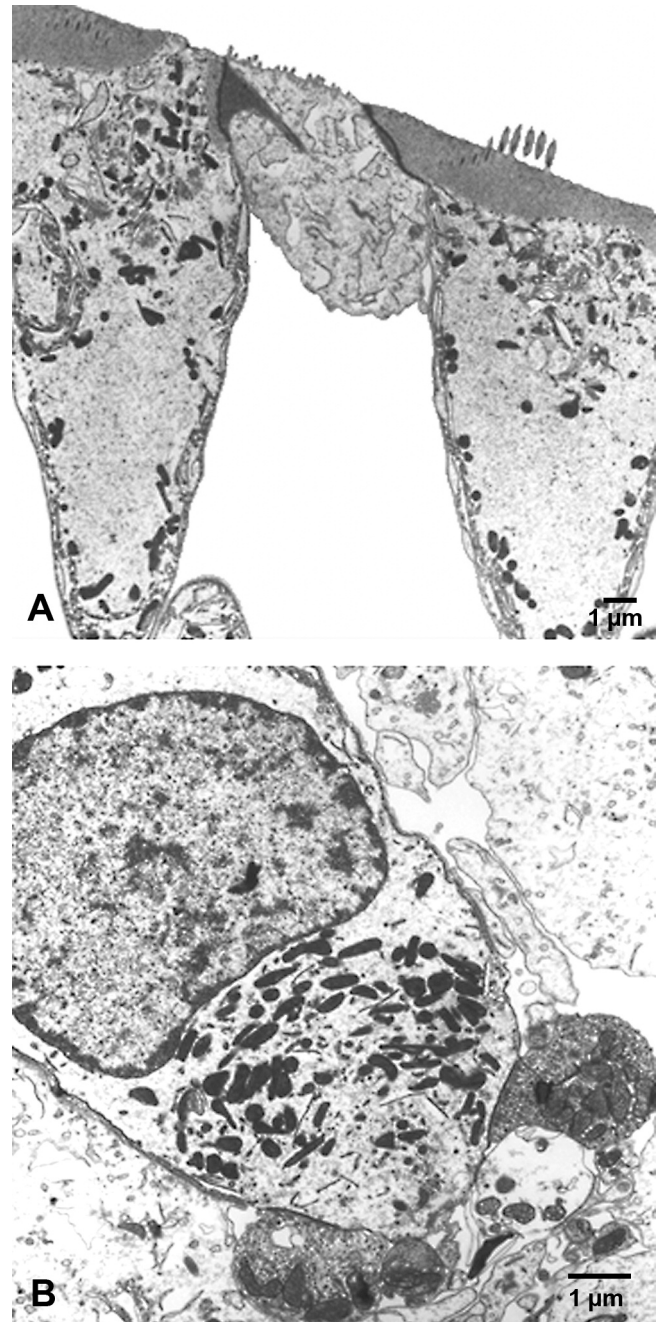


Fig. 3. Transmission electron micrographs of outer hair cells (OHC) from guinea pigs co-treated with cisplatin + CoA. **A.** OHC characterized by normal cellular ultrastructure similar to the untreated control group. **B.** Basal surface of a synapse showing typical, normal mitochondria.

examine the toxicity of cisplatin and the protective effect of CoA on cisplatin-induced cytotoxicity in OHC from guinea pigs. Our results showed that co-treatment with CoA significantly decreased the cytotoxic effects induced by cisplatin in both functional and ultrastructural parameters.

The increase in auditory thresholds and latencies as a result of the ototoxic effect of cisplatin has been reported in several other experimental studies (Yancey et al., 2012; Yumusakhuylyu et al., 2012; Hughes et al., 2014). As noted in the introduction, studies aimed at minimizing or preventing these ototoxic alterations have tested a number of drugs with protective actions. Our results with CoA show that auditory thresholds improved in comparison to most other drugs tested to date, with values similar to those in the control group. The same protective effect was reported after the administration of pantothenic acid (Ciges et al., 1996), thiosulfate (Berglin et al., 2011), dexamethasone (Daldal et al., 2007; Erdem et al., 2012), and resveratrol (Yumusakhuylyu et al., 2012; Olgun et al., 2014). However, unlike these studies, our results also show decreased N1 wave latency in group III (cisplatin + CoA) compared to group I (untreated control). This finding supports the protective role of CoA in the functional activity of OHC.

Our TEM observations of OHC show that a single high dose (10 mg/ml) of cisplatin during 7 days induced loss of the normal pattern of organization of the smooth endoplasmic reticulum, areas of edema, intracytoplasmic degeneration, mitochondrial vacuolation and loss of cristae. Previous studies in Wistar albino rats treated with cisplatin (16 mg/kg) reported ultrastructural alterations in OHC similar to those we saw in the present study (Gunes et al., 2011; Olgun et al., 2014; Kirkim et al., 2015). Although alterations in the cilia are a major feature of cisplatin-induced ototoxicity in studies based on scanning electron microscopy (Ciges et al., 1996; Waissbluth et al., 2013), our observations with TEM did not disclose severe damage to the cilia; in fact, in some samples the appearance of the cilia was compatible with normality. In addition, we saw no signs of alteration in the cuticular plates, although this cytotoxic effect was reported by others (van Ruijven et al., 2004, 2005). Also absent in our ultrastructural examination were nuclear alterations that might be related with processes of apoptosis (Calli et al., 2012; Olgun et al., 2014).

Further observations with TEM in guinea pigs co-treated with cisplatin and CoA revealed no alterations in the ultrastructure of OHC. This result is consistent with findings reported after the use of a number of compounds able to prevent ototoxic effects (Ciges et al., 1996; Daldal et al., 2007; Yumusakhuylyu et al., 2012; Olgun et al., 2014). In addition, the absence of ultrastructural alterations was associated with functional recovery after CoA co-administration with cisplatin.

Although the mechanism of action for the protective effect of CoA has not been entirely elucidated, this enzyme is known to play an important role in many biological mechanisms as an electron acceptor, by

accelerating enzyme-catalyzed biological oxidation. It may act by facilitating the removal of breakdown products of cisplatin, in a manner similar to thiosulfate (Otto, 1988), or by enhancing mitochondrial function by maintaining energy levels that favor cellular biochemical activities that are altered by the action of cisplatin.

Coenzyme A is well known to be involved in fatty acid metabolism and acetylcholine synthesis (Kageyama et al., 2011; Szutowicz et al., 2013). In this connection, fatty acid β -oxidation has been related with increased cisplatin cytotoxicity. Cell populations resistant to cisplatin cytotoxicity have been identified as having elevated levels of the enzymes involved in fatty acid β -oxidation (e.g. 2,4-dienoyl-CoA reductase and enoyl-CoA isomerase). In this connection the cholinergic nature of OHC is worth recalling, because normal OHC functioning is thought to be related with adequate CoA levels. In our experimental model of cisplatin ototoxicity, the pattern of latencies improved after CoA administration compared to untreated control animals; this finding suggests that in addition to its protective effect, CoA also potentiates the functional efficacy of OHC.

In conclusion, we analyzed the protective effects of CoA in OHC in the guinea pig cochlea and found that CoA significantly reduced the morphological alterations induced by cisplatin. Our results suggest that CoA has a protective effect against cisplatin-induced toxicity. Further studies are warranted to test whether CoA has potential clinical applications to decrease cisplatin-induced ototoxicity.

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