Cellular/Molecular

Role of the Copper Transporter, CTR1, in Platinum-Induced Ototoxicity

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The goal of this study was to determine the role of an influx copper transporter, CTR1, in the ototoxicity induced by cisplatin, a potent anticancer platinum analog used in the treatment of a variety of solid tumors. As determined through reverse transcriptase-PCR (RT-PCR), quantitative RT-PCR, Western blot, and immunohistochemistry, mouse CTR1 (Ctr1) was found to be abundantly expressed and highly localized at the primary sites of cisplatin toxicity in the inner ear, mainly outer hair cells (OHCs), inner hair cells, stria vascularis, spiral ganglia, and surrounding nerves in the mouse cochlea. A CTR1 substrate, copper sulfate, decreased the uptake and cytotoxicity of cisplatin in HEI-OC1, a cell line that expresses many molecular markers reminiscent of OHCs. Small interfering RNA-mediated knockdown of Ctr1 in this cell line caused a corresponding decrease in cisplatin uptake. In mice, intratympanic administration of copper sulfate 30 min before intraperitoneal administration of cisplatin was found to prevent hearing loss at click stimulus and 8, 16, and 32 kHz frequencies. To date, the utility of cisplatin remains severely limited because of its ototoxic effects. The studies described in this report suggest that cisplatin-induced ototoxicity and cochlear uptake can be modulated by administration of a CTR1 inhibitor, copper sulfate. The possibility of local administration of CTR1 inhibitors during cisplatin therapy as a means of otoprotection is thereby raised.

Introduction

The utility of the chemotherapeutic agent, cisplatin [$(NH_3)_2PtCl_2$] remains limited because of drug resistance and dose-limiting toxicities, particularly nephrotoxicity and ototoxicity (Blakley and Myers, 1993; McKeage, 1995). One-third of all cisplatin-treated cancer patients develop irreversible hearing loss (Li et al., 2004). Cisplatin ototoxicity initially presents as tinnitus and sensorineural hearing loss beginning at high frequencies, inconsequential for speech perception, but eventually approaching lower frequencies necessary for speech perception (Piel et al., 1974). Hearing threshold elevation occurs in 60–80% of all cisplatin-treated patients. Approximately 15% of patients suffer significant functional hearing loss (McKeage, 1995). A hallmark of cisplatin toxicity is loss of outer hair cells (OHCs) beginning from the cochlear base. A recent study suggests additionally the involvement of stria vascularis and spiral ganglion (Cardinaal et al., 2000).

Current knowledge implicates reactive oxygen species (ROS) and overexpression of high mobility group protein 1 (HMG1) in cisplatin ototoxicity (Rybak et al., 2007). Use of antioxidants (Muldoon et al., 2000; Wimmer et al., 2004) or antiapoptotic agents (Wang et al., 2004) do not, however, exhibit any clinically useful otoprotection. Cisplatin ototoxicity is currently countered by replacement with less potent nontoxic chemotherapeutics or accepted as a health outcome in exchange for successful cancer treatment. A pressing need exists for chemopreventive strategies that can predict or alleviate cisplatin-induced ototoxicity.

This study sought to examine the role of uptake transporters in cisplatin ototoxicity, focusing particularly on the organic cation transporter OCT2 and on the copper transporter CTR1, previously implicated in cisplatin-induced nephrotoxicity (Ciarimboli et al., 2005; Pabla et al., 2009). (Ctr1 and CTR1 designate the mouse and human transporters, respectively.) The organic cation transporter is a membrane protein that transports cations such as tetraethylammonium, MPP^+, metformin, and paraquat (Jonker and Schinkel, 2004; Wright, 2005). On partial solvolization, cisplatin forms $[(NH_3)_2PtCl(H_2O)]^+$ (monoaqua complex), which can be transported by OCT2 (Fig. 1). The diabetic kidney carrying reduced levels of OCT expression is resistant to cisplatin toxicity (Ciarimboli et al., 2005). The human copper transporter, CTR1, consists of three transmembrane domains oligomerizing to a functional trimer (Lee et al., 2001). The expression of CTR1 in various cancer cell lines affects cellular accumulation and sensitivity to cisplatin treatment (Ishida et al., 2002; Zisowsky et al., 2007).

Described herein are our efforts at mapping the cochlear expression and localization of OCT2 and Ctr1 in mouse. Experiments involving the cochlear cell line HEI-OC1 and *in vivo* experiments with CTR1 substrates and inhibitors serve to examine functional consequences of CTR1-mediated cisplatin uptake

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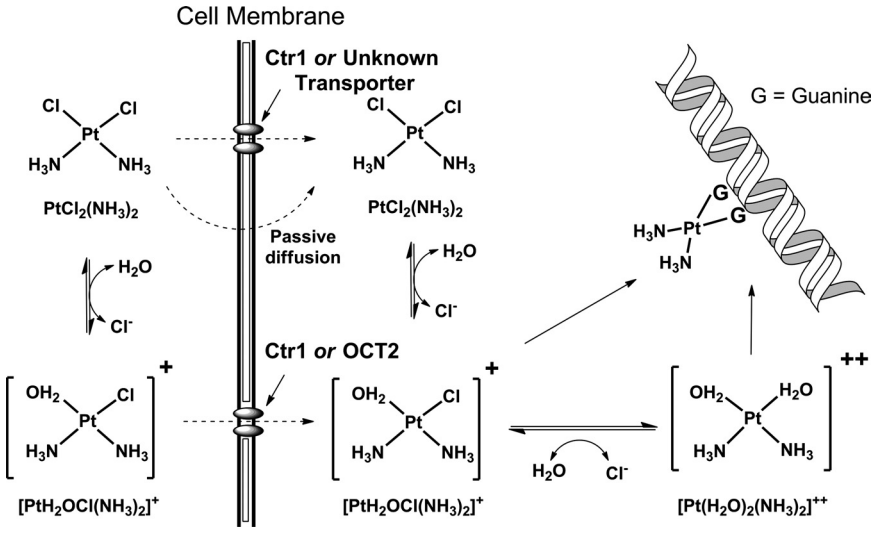


Figure 1. Schematic representation of cisplatin uptake and the chemistry involved in its mechanism of action.

and establish its role in cisplatin ototoxicity. Ctr1, and not OCT2, was found to be expressed at the primary site of cisplatin-induced ototoxicity (i.e., hair cells of organ of Corti). Importantly, knockdown of Ctr1 by small interfering RNA (siRNA) reduced cisplatin uptake in the HEI-OC1 cell line. Utilization of a CTR1 substrate, copper(II), along with cisplatin demonstrated a protective effect *in vitro* and *in vivo*, presumably by competing with cisplatin for uptake. These results suggest a pathway for cisplatin ototoxicity involving the uptake of cisplatin into cochlea via Ctr1, and thus presenting a promising otoprotective strategy.

Materials and Methods

Reagents

Antibodies for immunohistochemistry, immunofluorescence, and Western blotting were purchased from the following sources: rabbit polyclonal anti-Ctr1 antibody from Novus Biologicals, rabbit polyclonal anti-OCT2 antibody from Alpha Diagnostic International, myosin VIIa (Proteus Biosciences), and mouse monoclonal anti-β-actin antibody from Santa Cruz Biotechnology. Horseradish peroxidase-conjugated anti-rabbit (sc-2004; Santa Cruz Biotechnology) Ig was used as the secondary antibody. Lipofectamine RNAiMAx, SuperScript III, and hygromycin B were from Invitrogen. The cell culture media DMEM, Opti-MEM, and fetal bovine serum (FBS) were from the Cell Culture Facility at University of California, San Francisco (San Francisco, CA). Cisplatin and copper sulfate were purchased from Sigma-Aldrich.

Animals and cell lines

The FVB and C57BL/6 wild-type mice (3–4 weeks) were purchased from Charles River Laboratories. All experimental procedures and animal handling were executed in accordance with national ethics guidelines, approved and complied with all protocol requirements at the University of California, San Francisco (Institutional Animal Care and Use Committee). The cochlear cell line, HEI-OC1, was kindly provided by Dr. Federico Kalinec (House Ear Institute, Los Angeles, CA). The cells were cultured in DMEM-H21 containing 10% FBS and were incubated at 33°C in a humidified atmosphere containing 10% CO_2. Human embryonic kidney 293 (HEK293) cells transfected with pcDNA5/FRT (Invitrogen) vector containing the full-length human CTR1 cDNA (HEK-CTR1) and with the empty vector (HEK-EV) were established using Lipofectamine 2000 (Invitrogen) as per the manufacturer's instructions. Transfected HEK293 cells were maintained in DMEM-H21 containing 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 750 μg/ml hygromycin B at 37°C in a humidified atmosphere containing 5% CO_2.

Reverse transcriptase-PCR

Total RNA was extracted from the cochleae from male/female mice and HEI-OC1 cells using the Purelink RNA mini kit (Invitrogen) as per the manufacturer's instructions. Reverse transcription PCR of RNA samples was performed with Superscript III (Invitrogen) using oligo-dT_{20} primers. Two microliters of the reverse transcriptase (RT) reaction product was used for subsequent PCRs (TaqDNA Polymerase; Invitrogen) of 35 cycles with the following parameters: 94°C for 30 s, 60°C for 45 s, 72°C for 1 min, followed by a final extension of 72°C for 10 min and storage at 4°C. Primers were designed to amplify a unique sequence of mouse Ctr1, OCT1, OCT2, and OCT3, each spanning intron–exon boundaries to ensure that no genomic DNA was amplified. The PCR primers that were used for mouse were as follows: Ctr1 (GenBank accession number NM_175090), forward, CATGATGATGATGCCTATGACC, and reverse, CAGCATCTGCTGCCCAAC, were designed to amplify a 268 bp fragment; OCT1 (NM_009202), forward, ACACCTTGATCACAGAGTTTG, and reverse, CAAGGCACATCATCTTCAGGT, were designed to amplify a 306 bp fragment; OCT2 (NM_013667), forward, TCTTGATGTACAATTGGTTCACG, and reverse, AACCACAGCAAATACGACCAG, primers were designed to amplify a 461 bp fragment; and OCT3 (NM_011395), forward, TTATGTTTGCTTGGTTCACGAG, and reverse, AGGATCCCAAAGATGATCAGAG, primers were designed to amplify a 457 bp fragment. Analysis of each PCR sample was then performed on 2% agarose gels containing 0.5 μg/ml ethidium bromide. Gels were visualized using a digital camera and an image processing system (Kodak).

Quantitative PCR

Real-time PCR was performed using Taqman Universal Master Mix (Applied Biosystems). Primer and probe sets for each gene were Assays-on-Demand purchased from Applied Biosystems. Reactions were run on an ABI Prism 7700 instrument, and cycling conditions were as follows: 95°C for 20 s followed by 60 cycles of 95°C for 3 s and 60°C for 30 s. The amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was performed as an internal control. The expression of specific gene of interest normalized to GAPDH is reported as the level relative to the expression of the lowest expressing transporter, OCT3, in that particular sample.

Immunohistochemistry and immunofluorescence

The male FVB mice (3–4 weeks of age) were anesthetized and their cochleae were isolated, dissected, perfused through oval and round windows by 2% paraformaldehyde in 0.1 M phosphate buffer (PB) at pH 7.4, and incubated in the same fixative for 2 h. On fixation, the cochleae were rinsed with PB and immersed in 5% EDTA in 0.1 M PB for decalcification. When the cochleae were completely decalcified (~2 d), they were incubated overnight in 30% sucrose for cryoprotection. The cochleae then were embedded in OCT TissueTek Compound (Miles Scientific). Tissues were cryosectioned at 10–12 μm thickness for immunohistochemistry, mounted on Superfrost microscope slides (Erie Scientific), and stored at −20°C until their use.

For immunohistochemical staining of the mouse cochlea, rabbit polyclonal antibodies against Ctr1 (Novus Biologicals) and OCT2 (Alpha Diagnostic) were used. After incubation of the slides at 37°C for 30 min, antigen retrieval was performed using 0.4% pepsin (Sigma-Aldrich) in 0.01N hydrochloric acid for 5 min at 37°C. The sections were rinsed twice for 5 min in 0.1 M PBS, pH 7.4, and then preincubated for 1 h in 0.3% Triton X-100 and 30% normal goat serum before incubating with rabbit anti-Ctr1 (1:500) or rabbit anti-OCT2 (1:250) diluted in the blocking serum overnight at 4°C. The slides were then rinsed (two times for 10 min each time) and Vector biotinylated goat anti-rabbit Ig was used as the

secondary antibody followed by Vector ABC (Vector Laboratories). Peroxidase activity was identified by reaction with 3,3′-diaminobenzidine tetrahydrochloride tablets (Sigma-Aldrich). Sections were mounted in equal parts of glycerol and PBS buffer before being coverslipped. Slides treated with the same technique, but without incubation with the primary antibody, were used as the negative control.

Immunofluorescence studies were conducted on whole-mount cochleae prepared as described below (see Hair cell counts). A primary antibody against a hair cell-specific marker, myosin VIIa, was used at a dilution of 1:50 in PBS. After an overnight incubation at 4°C, the sections were rinsed twice for 10 min with PBS and then incubated for 2 h in goat anti-rabbit IgG conjugated to Cy3 diluted to 1:2000 in PBS. The sections were rinsed in PBS twice for 10 min, and mounted in a 1:1 mixture of PBS buffer and glycerol before being coverslipped. Slides treated with the same technique but without incubation with the primary antibody were used as controls. Slides were observed under an Olympus microscope with confocal immunofluorescence.

Western blot analysis

Total protein extract was prepared from male/female mice cochleae using a lysis buffer containing the following: 20 mM Tris HCl, pH 7.5–8.0, 150 mM NaCl, 0.5% sodium deoxycolate, 1% Triton X-100, 0.1% SDS, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor mixture. The HEI-OC1 cell pellet was lysed using CellLytic M cell lysis buffer (Sigma-Aldrich) at 4°C for 10 min. The cochlear and HEI-OC1 cell homogenate was spun for 10 min at 14,000 rpm at 4°C. The protein concentration in the supernatant was determined by the BCA protein assay. Approximately 50 μg of the supernatant was fractioned by SDS-PAGE and transferred onto a polyvinylidene difluoride membrane. The blots were incubated for 1 h in a blocking buffer containing 5% nonfat dry milk in TBS and then incubated overnight at 4°C with anti-Ctr1 or OCT2 antibodies used for immunohistochemistry and with anti-β-actin antibody, as a loading control, diluted in TBS-T buffer containing 2% milk (dilutions: Ctr1, 1:1000; OCT2, 1:500; β-actin, 1:5000). The blots were washed three times with TBS-T for 15 min each and incubated for 2 h at room temperature with the secondary antibody (goat anti-rabbit IgG peroxidase conjugate; Santa Cruz Biotechnology; 1:5000) in TBS-T buffer containing 3% milk. The blots were washed again with TBS-T and visualized with the ECL Western blotting detection reagent (GE Healthcare).

Cytotoxicity studies

The cytotoxicity of cisplatin was measured by standard MTT assays in 96-well plates as previously described (Zhang et al., 2006) in HEK293 and HEI-OC1 cells. After seeding the cells at the desired density and overnight incubation, cisplatin with or without a CTR1 competitive inhibitor, copper sulfate (1 mM), was then added to the culture medium to various concentrations. After 7 h of drug exposure, the drug-containing medium was replaced with fresh, drug-free medium, and the incubation was allowed to continue for 72 h after the addition of the drugs. At the end of the incubation, 20 μl of MTT stock solution (5 mg/ml) was added to each well. After additional incubation for 3 h at 37°C, the MTT reaction medium was discarded and the purple-blue MTT formazan crystals were dissolved by the addition of 100 μl of 0.1N HCI in isopropanol. The optical density, which is a reflection of the mitochondrial function of the viable cells, was read directly with a microplate reader (Versamax; Molecular Devices) at 580 nm and a reference wavelength of 680 nm. Concentration response graphs were generated for each drug using GraphPad Prism software (GraphPad Software). These graphs were analyzed using a curve fit for sigmoid dose–response, and IC_{50} values were derived. Results are expressed as mean IC_{50} with the SEM.

Cellular accumulation of platinum

The cellular accumulation of platinum was determined as previously described (Zhang et al., 2006). Briefly, the cells were seeded in poly-D-lysine-coated 12-well plates. After an overnight incubation, the cells were treated with 10 μM concentration of cisplatin in antibiotic-free culture medium in the presence or absence of copper sulfate at 37°C in 5% CO_2 for 2 h. At the end of incubation, the cellular uptake was terminated by washing three times with ice-cold PBS, harvested, and pelleted by centrifugation. Cell pellets were solubilized in 70% nitric acid at 65°C for 3 h and diluted with distilled water containing 10 ppb of iridium (Sigma-Aldrich) and 0.1% Triton X-100 to adjust the nitric acid concentration to 7%. Platinum content was then determined by inductively coupled plasma mass spectrometry (ICP-MS) in the Analytical Facility at University of California, Santa Cruz (Santa Cruz, CA). Cellular platinum uptake was normalized to the protein content determined by BCA protein assay.

Cisplatin uptake in HEI-OC1 cells was performed after siRNA-mediated Ctr1 knockdown using Lipofectamine RNAiMax (Invitrogen). Briefly, the cells were transfected with siRNA-lipofectamine RNAiMax complexes of Ctr1 and negative control siRNA (Applied Biosystems) in a poly-D-lysine-coated 24-well plate per the manufacturer's instructions. The percentage knockdown of Ctr1 was determined 48 h after transfection in comparison with negative control transfected cells. Cisplatin uptake was performed at this time using conditions described before.

To examine cochlear uptake of cisplatin *in vitro*, cochlear organs of Corti from 12-d-old male mice were dissected in sterile saline solution composed of the following (in mM): 6.8 KCl, 144 NaCl, 0.9 $MgCl_2$, 1.3 $CaCl_2$, 0.7 NaH_2PO_4, 5.6 D-glucose, 10 HEPES. The cochlea was isolated from the surrounding temporal bone. The isolated and intact organ of Corti (basal, mid, and apical turns) was progressively freed after removal of the lateral half of the cochlea otic capsule. The organ of Corti was fully exposed at this point and remained in the medial or bilateral half of the cochlear capsule. This organ of Corti was then immediately placed in an antibiotic-free medium (DMEM-H21) and incubated with cisplatin (10 μM) in the presence or absence of copper sulfate (1 mM) at 37°C for 2 h to determine the rate of platinum uptake. The net platinum concentrations, determined through aforementioned methods, were normalized to the weight of dry tissue.

Platinum-DNA adduct formation

The platinum content associated with genomic DNA was determined as previously described (Zhang et al., 2006). Briefly, the cells were seeded in 60 mm tissue culture dishes. After overnight incubation, cells were treated with cisplatin (10 μM) in antibiotic-free culture medium with or without an OCT inhibitor at 37°C in 5% CO_2 for 2 h. At the end of incubation, the cells were washed with ice-cold PBS, harvested, and pelleted. Genomic DNA was isolated from the cell pellets using Wizard Genomic DNA Purification Kit (Promega) according to the manufacturer's protocol. The DNA-bound platinum concentration was determined by ICP-MS, which was normalized to total DNA content (absorption spectrometry at 260 nm).

In vivo *study design*

To determine the effect of intratympanic administration of copper sulfate on intraperitoneal cisplatin treatment, the study was divided in three phases. In phases 1 and 2, ototoxic and nonototoxic doses of intraperitoneal cisplatin and intratympanic copper sulfate were determined, respectively. The animals were anesthetized before administration of extraneous agents, by intraperitoneal injection of a mixture of ketamine hydrochloride (Ketaset; 100 mg/kg) and xylazine hydrochloride (Xyla-Ject; 10 mg/kg). Body temperature was maintained with the aid of a heating pad. The depth of anesthesia was determined by measurement of pedal reflex.

Eighteen C57BL/6 male mice were divided into six treatment groups of cisplatin (0, 10, 14, 18, 20, and 22 mg/kg) and copper sulfate (0, 0.025, 0.04, 0.05, 0.1, and 0.5 mg/kg) for phases 1 and 2, respectively. For each group, pretreatment acoustic brainstem response (ABR) thresholds were determined on day 1 at click, 8, 16, 32 kHz frequencies. Determination of changes in ABR thresholds of an individual animal with respect to the control was performed after 72 h of cisplatin administration. Based on ABR results and survival data, a dose of 20 mg/kg cisplatin and 0.025 mg/kg for copper sulfate was chosen for the phase 3 experiments. Effect of copper sulfate on cisplatin-induced ototoxicity was determined 72 h after cisplatin administration. In this study, 15 C57BL/6 male mice were randomized into three groups of 5 animals each.

Group 1 (cisplatin and intratympanic water). Under an operating microscope, an intratympanic injection of water (~5 μl) was administered slowly through a myringotomy in the anterosuperior quadrant, with a 28

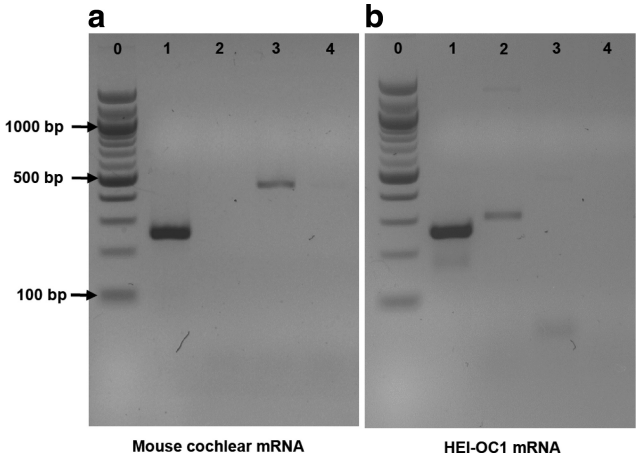


Figure 2. Expression analysis of candidate transporters for cisplatin in mouse cochlear tissue (***a***) and HEI-OC1 (***b***) by RT-PCR. The mRNA expression of Ctr1, OCT1–3 transporters was detected as described in Materials and Methods. Lanes 0 – 4 (product size): 0, 100 bp marker; 1, Ctr1 (268 bp); 2, OCT1 (306 bp); 3, OCT2 (461 bp); 4, OCT3 (457 bp).

gauge dental needle to fill the middle ear cavity. After retaining the animal in the same position for 30 min, cisplatin (20 mg/kg) in saline containing 5% DMSO was administered intraperitoneally as a slow infusion.

Group 2 (intratympanic copper sulfate 30 min before cisplatin). The procedure described for group 1 was performed except that an intratympanic injection of copper sulfate (0.025 mg/kg) was administered instead of water. Thereafter, 30 min after the copper sulfate treatment, cisplatin (20 mg/kg) was administered intraperitoneally as a slow infusion.

Group 3 (intratympanic copper sulfate 4 h before cisplatin). The procedure described for group 2 was performed. Here, 4 h after the copper sulfate injection, cisplatin (20 mg/kg) was administered intraperitoneally as a slow infusion.

ABR measurements

The animals were anesthetized with ketamine hydrochloride and xylazine before testing. Evoked acoustic brainstem response thresholds were differentially recorded from the scalp of the mice as described previously (Akil et al., 2006). The sound stimuli used included click (5 ms duration) and tone pips at 8, 16, and 32 kHz (10 ms duration). Measurements were done using the TDT BioSig III system (Tucker-Davis Technologies). Computerized analysis of stored waveforms for ABR threshold (Matlab software; The MathWorks) was also performed. The mean value of thresholds checked by visual inspection and computer analysis was defined as the ABR threshold at each stimulus and was compared among different treatment groups.

Histopathology with light microscopy

Following our previous protocols (Akil et al., 2006), the C57BL/6 mice of groups 1 and 2 were anesthetized and the cochleae were isolated, dissected, perfused through the round window with 2% paraformaldehyde and 2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, and incubated in the same fixative overnight at 4°C. The cochleae were subsequently rinsed with 0.1 M PB and postfixed in 1% osmium tetroxide for 1 h. The bone surrounding the cochlea was thinned and the cochlea subsequently immersed in 5% EDTA (0.2 M). The decalcified cochleae were dehydrated in ethanol and propylene oxide, whereupon they were embedded in Araldite 502 resin (Electron Microscopy Sciences) and sectioned at 5 μm. Sections were stained with toluidine blue and mounted with Permount (Thermo Fisher Scientific) on microscope slides.

Hair cell counts

The cochleae from C57BL/6 mice used in groups 1 and 2 were perfused with 4% PFA in 0.1 M PBS, pH 7.4, and stored in the fixative overnight at 4°C. The cochleae were subsequently rinsed with PBS three times for 10 min each, and then decalcified with 5% EDTA in 0.1 M PBS for 3 d. The otic capsule, the lateral wall, tectorial membrane, and Reissner's membrane were removed in that order. The remaining organ of Corti was stained

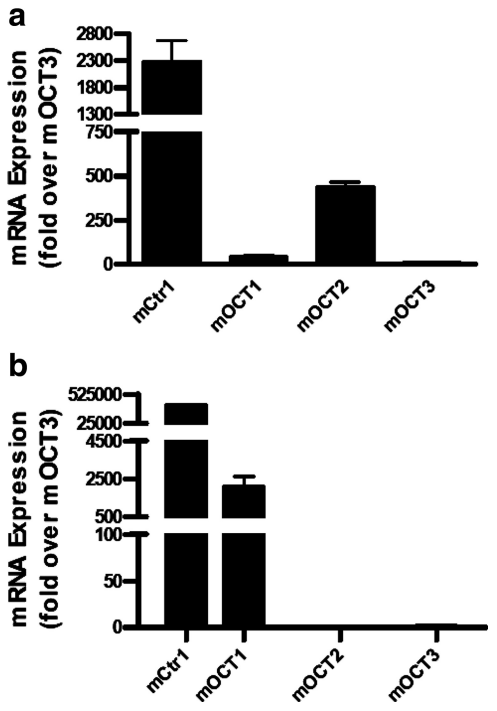


Figure 3. Quantitation of mRNA expression levels of candidate transporters for cisplatin in mouse cochlear tissue (***a***) and HEI-OC1 (***b***). The qRT-PCR analysis was performed on the total RNA extracted using the primer probe sets for the individual transporter. The mRNA expression of the individual transporter is normalized to the level of GAPDH level in that sample and expressed as mean ± SEM of three individual experiments.

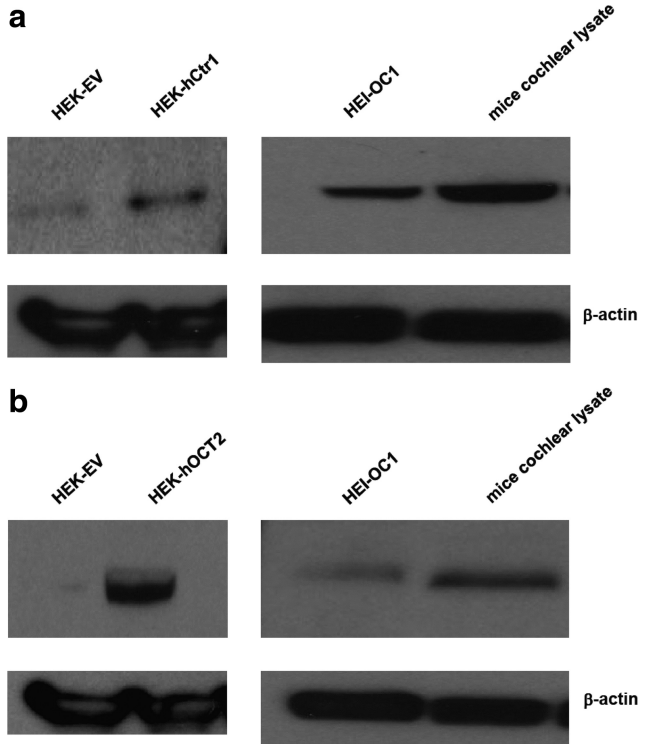


Figure 4. Western blot expression analysis of the copper transporter Ctr1 (***a***) and organic cation transporter 2 (OCT2) (***b***) in HEI-OC1 cell line and mouse cochlear lysate. Ctr1- and OCT2-transfected HEK293 cell lines were used as positive controls. The presence of Ctr1 and OCT2 in these samples was detected as a band at ~25 and ~75 kDa, respectively.

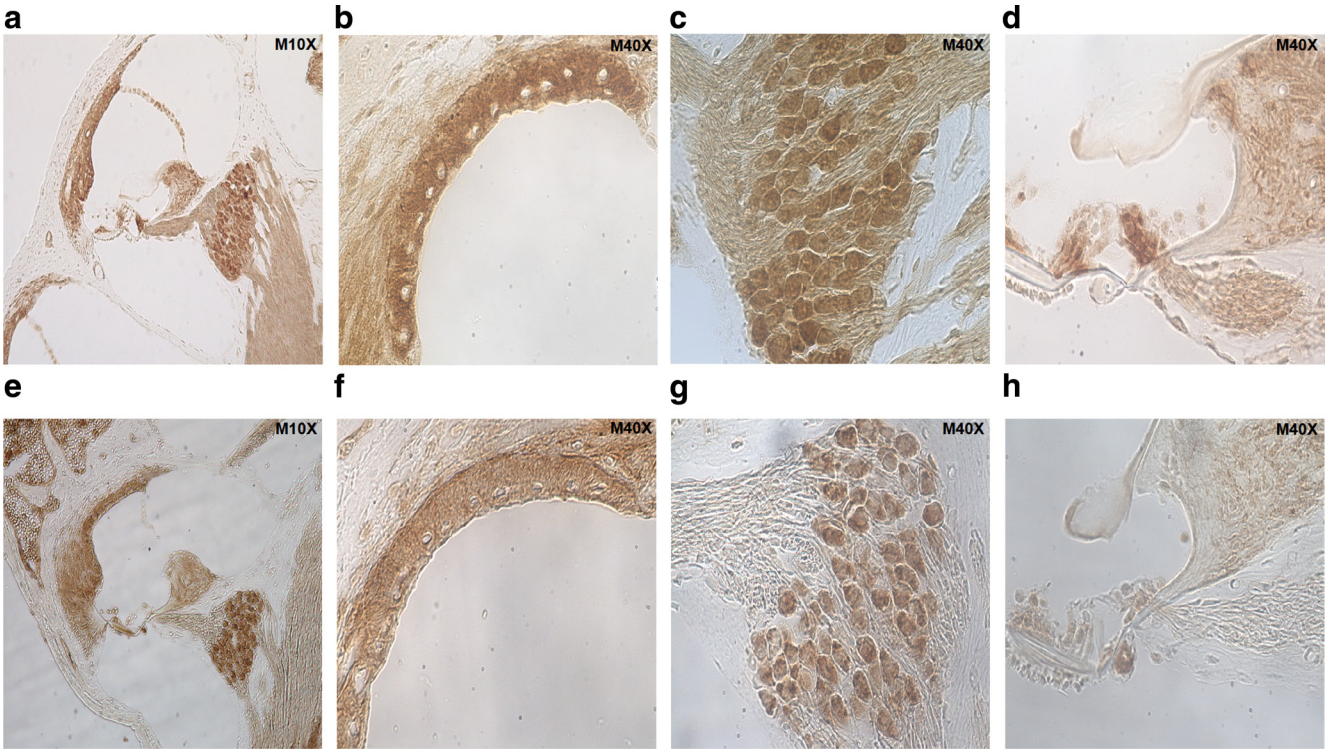
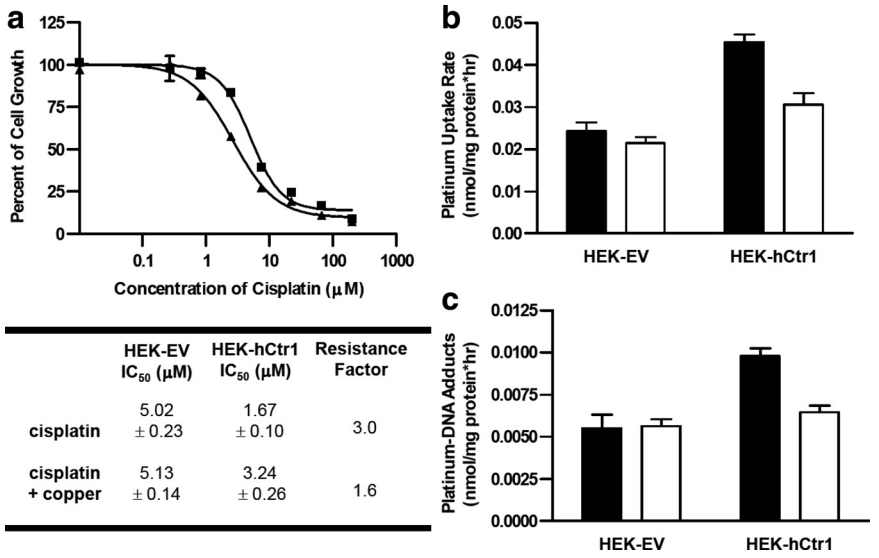


Figure 5. Immunohistochemical detection of the copper transporter Ctr1 (***a–d***) and organic cation transporter 2 (OCT2) (***e–h***) in mouse cochlea. The localization of these transporters in specific portions of cochlea are provided with higher magnification images of stria vascularis (***b***, ***f***), spiral ganglion (***c***, ***g***), and organ of Corti (***d***, ***h***). The presence of Ctr1 but not of OCT2, in organ of Corti, specifically in outer and inner hair cells stresses its role in cisplatin uptake and hence the resulting ototoxicity.

with rhodamine–phalloidin (stock solution of 200 U/ml methanol diluted 1:100 in PBS) for an hour. The whole mount was then rinsed with PBS and further dissected into a surface preparation (microdissected into individual turns) and mounted on glass slides in a mounting solution. Hair cells in the organ of Corti were visualized under a microscope equipped with epifluorescence, using a 40× objective. To quantify hair cell loss in the cochlea after the various treatments, Inner hair cells and OHCs were counted over each 0.1 mm sectional distance along the cochlea.

Data analysis

Data were analyzed statistically by unpaired or paired Student's *t* tests, as appropriate. Statistical significance was set at $p < 0.05$. For the cytotoxicity experiments, concentration response graphs were generated for each drug using the sigmoidal dose–response model in GraphPad Prism software (GraphPad Software). The IC_{50} values derived from these graphs are expressed as mean IC_{50} with SEM. ANOVA was used to compare within and between treatment groups for our *in vivo* studies in mice to derive statistical significance.



	HEK-EV IC_{50} (μM)	HEK-hCtr1 IC_{50} (μM)	Resistance Factor
cisplatin	5.02 ± 0.23	1.67 ± 0.10	3.0
cisplatin + copper	5.13 ± 0.14	3.24 ± 0.26	1.6

Figure 6. Cisplatin cytotoxicity (***a***), cellular accumulation rate (***b***), and platinum-DNA adducts (***c***) in hCtr1-transfected HEK cells. ***a***, The IC_{50} values of cisplatin in hCtr1-transfected (▲) and EV-transfected (■) HEK293 cells were determined by the standard MTT assay with drug exposure for 7 h as described in Materials and Methods. Increase in cisplatin IC_{50} was observed in the presence of copper sulfate (1 mM). ***b***, ***c***, hCtr1- and EV-transfected HEK cells were exposed to cisplatin (10 μM) in the presence (white bars) and absence (black bars) of copper sulfate (1 mM) for 2 h at 37°C in an antibiotic-free medium, and platinum concentration inside the cells and bound to DNA was determined as described in Materials and Methods. Data are expressed as the mean ± SEM of three independent experiments.

Results

Ctr1 was expressed in cochlea and was localized to the base of outer hair cells, inner hair cells, spiral ganglion, and stria vascularis of the mouse

RT-PCR detected Ctr1 and OCT2 transporters in mouse cochlea (Fig. 2). Expression levels of Ctr1 mRNA were the highest relative to all of the other transporters studied in mouse cochlear tissue and HEI-OC1 cells. OCT2 mRNA was detected in mouse cochlear tissue but not in HEI-OC1. HEI-OC1 tested positive for OCT1 mRNA. Quantitation by real-time PCR (qRT-PCR) (Fig. 3) on cochlear tissue and HEI-OC1 also indicated that Ctr1 expression levels were the highest relative to the other transporters

studied. Western blot analyses (Fig. 4) were indicative of the abundance of the Ctr1 protein in both cochlear lysates and HEI-OC1, whereas the OCT2 protein was unique to cochlear lysates.

The localization of Ctr1 and OCT2 in the mouse cochlea was determined. The primary sites for cisplatin toxicity, which include the outer and inner hair cells in the cochlea, exhibited high expression levels of Ctr1 (Fig. 5*d*; supplemental Fig. S1, available at www.jneurosci.org as supplemental material). OCT2 was absent in the hair cells of the organ of Corti (Fig. 5*h*; supplemental Fig. S1, available at www.jneurosci.org as supplemental material). Secondary sites (spiral ganglion and stria vascularis), which represent sites less commonly affected by cisplatin, exhibited expression of both Ctr1 (Fig. 5*a–c*) as well as OCT2 (Fig. 5*e–g*). These results suggested that Ctr1 and not OCT2 could potentially mediate the transport of cisplatin into the hair cells of the organ of Corti. In contrast, cisplatin-induced damage to other parts of the inner ear could be attributed to its uptake by both Ctr1 and OCT2.

CTR1 enhanced the cytotoxicity of cisplatin

In a standard MTT assay (7 h exposure), cisplatin had an IC_{50} value of 1.67 ± 0.10 μM in HEK293 cells transfected with human CTR1 (HEK-CTR1), whereas it was 5.02 ± 0.23 μM (threefold higher) in the corresponding empty vector (HEK-EV) cells (Fig. 6*a*) ($p < 0.01$). Coincubation of the CTR1 substrate Cu^{2+} (as $CuSO_4$, 1 mM) reduced the cytotoxic potency of cisplatin in HEK-CTR1 cells (control vs $CuSO_4$ treated, 1.67 ± 0.10 vs 3.24 ± 0.26 μM; $p < 0.05$) while not affecting the potency of cisplatin in HEK-EV cells (control vs $CuSO_4$ treated, 5.02 ± 0.23 vs 5.13 ± 0.14 μM; $p > 0.05$) (Fig. 6*a*). Under these conditions, $CuSO_4$ itself exhibited no cytotoxicity up to concentrations of 10 mM (data not shown).

CTR1 enhanced the cellular uptake and DNA adduct formation rate of cisplatin

Cisplatin was found to accumulate 1.9-fold faster over a period of 2 h in HEK-CTR1 cells [0.045 ± 0.003 nmol/(mg protein · h)] compared with HEK-EV cells [0.024 ± 0.004 nmol/(mg protein · h)] ($p < 0.001$) (Fig. 6*b*). Coincubation of $CuSO_4$ (1 mM) reduced the accumulation of platinum in HEK-CTR1 cells [control vs $CuSO_4$ treated, 0.045 ± 0.003 vs 0.031 ± 0.005 nmol/(mg protein · h); $p < 0.001$] (Fig. 6*b*) with little effect in HEK-EV cells [control vs $CuSO_4$ treated, 0.024 ± 0.004 vs 0.021 ± 0.002 nmol/(mg protein · h); $p > 0.05$] (Fig. 6*b*). A parallel experiment to examine the effect of $CuSO_4$ on the cisplatin uptake function of OCT2 indicated the presence of no such interaction (data not shown).

A similar trend was observed after comparison of concentrations of platinated DNA in HEK-CTR1 versus HEK-EV (Fig. 6*c*). The platinum-DNA adduct concentration after 2 h exposure to cisplatin in HEK-CTR1 cells was 9.799 ± 0.447 pmol/(mg DNA · h), 1.78-fold higher than that in HEK-EV cells [5.501 ± 0.793 pmol/(mg DNA · h); $p < 0.001$] (Fig. 6*c*). This platinum-DNA adduct formation was inhibited by $CuSO_4$ in HEK-CTR1 [control vs $CuSO_4$ treated; 9.799 ± 0.447 vs 6.44 ± 0.389 pmol/(mg DNA · h); $p < 0.001$] but again, to a very little extent in HEK-EV cells [control vs $CuSO_4$ treated; 5.50 ± 0.793 vs 5.62 ± 0.407 pmol/(mg DNA · h); $p > 0.05$] (Fig. 6*c*).

Results of the cellular uptake experiments demonstrated that CTR1 contributes to increased cellular uptake of cisplatin. Inhibition of this effect by the substrate of CTR1, Cu^{2+}, suggested that this involvement was specific to the presence of the CTR1-mediated molecular uptake mechanism. Results of the platinum-DNA adduct formation measurements demonstrated that CTR1 promoted the uptake of platinum into the interior of the cell in a form that was capable of DNA adduct formation and that DNA-adduct formation, a molecular mechanism for cisplatin cytotoxicity varied in direct proportion with the uptake of cisplatin into the cell.

Table 1. Effect of copper sulfate on cytotoxicity and cellular uptake of cisplatin in HEI-OC1 cells

Cytotoxicity[a] IC_{50} (μM)		
Cisplatin	10.16 ± 1.745	$p < 0.01$
Cisplatin + copper sulfate (1 mM)	32.27 ± 4.184	
Cellular uptake rate[b] (pmol/mg protein · h)		
Cisplatin	0.361 ± 0.027	$p < 0.05$
Cisplatin + copper sulfate (1 mM)	0.219 ± 0.030	

[a]Increase in IC_{50} value of cisplatin in HEI-OC1 cell line in the presence of copper. The cytotoxicity of cisplatin in HEI-OC1 cells was determined by MTT assay in which cells were exposed to cisplatin for 7 h in the presence or absence of copper sulfate (1 mM). The seeding density was at 5000 cells/well. The data are represented as the mean ± SEM of three independent experiments.

[b]Cellular accumulation of cisplatin in HEI-OC1 cell line in the presence of copper sulfate. HEI-OC1 cells were incubated in an antibiotic-free medium containing 10 μM cisplatin at 37°C and 5% CO_2 for 2 h in the presence and absence (black bars) of copper sulfate (1 mM) after which platinum concentration inside the cells was determined as described in Materials and Methods. Data are represented as the mean ± SEM of three independent experiments.

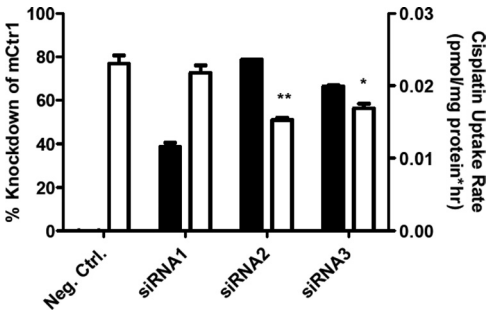


Figure 7. Reduced uptake of cisplatin after siRNA-mediated knockdown of Ctr1 in HEI-OC1 cell line. HEI-OC1 cells were transfected with siRNAs designed against Ctr1 in a 24-well plate using RNAiMax as a transfection reagent and total RNA was isolated 48 h after transfection. The percentage knockdown of Ctr1 was determined by qRT-PCR compared with negative control siRNA-transfected HEI-OC1 cells (black bars). Two days after the siRNA-mediated Ctr1 knockdown, HEI-OC1 cells were incubated with cisplatin (10 μM) for 2 h at 37°C and platinum accumulation was determined as described in Materials and Methods (white bars). The data are represented as the mean ± SEM of three independent experiments (*$p < 0.05$, **$p < 0.01$).

Ctr1 enhanced the cytotoxicity and cellular uptake of cisplatin in HEI-OC1

Cell death of HEI-OC1 caused by cisplatin treatment was measured by the MTT assay (Table 1). $CuSO_4$ (1 mM) reduced the cytotoxicity of cisplatin in HEI-OC1 [control vs copper sulfate treated; 10.2 ± 1.75 vs 32.3 ± 4.18 μM; $p < 0.01$]. These results were in agreement with the copper-mediated reduction in cisplatin uptake in this cell line [control vs $CuSO_4$ treated; 0.361 ± 0.027 vs 0.219 ± 0.030 nmol/(mg protein · h); $p < 0.05$]. Sublethal toxicity was not apparent on morphological examination of HEI-OC1 cells exposed to $CuSO_4$, which appeared normal after exposure to copper sulfate (supplemental Fig. S2, available at www.jneurosci.org as supplemental material).

Direct evidence of the role of Ctr1 in cisplatin uptake was obtained by performing the uptake experiment of cisplatin in Ctr1-siRNA-treated HEI-OC1 cells. The efficiency of siRNA-mediated Ctr1 knockdown was determined 48 h after transfection compared with that in the negative control siRNA transfected HEI-OC1 cells (Fig. 7). Three different siRNAs were used for Ctr1 knockdown, and the resultant decrease in Ctr1 expression was variable in all three siRNAs (percentage knock-

down using siRNA1, 38.52 ± 3.28; siRNA2, 78.64 ± 1.93; siRNA3, 66.35 ± 1.10). The cisplatin accumulation was reduced in Ctr1-knocked-down HEI-OC1 cells with highest reduction observed with siRNA2, which showed maximum knockdown of Ctr1 [negative control vs siRNA1 vs siRNA2 vs siRNA3 treated; 0.023 ± 0.001 vs 0.022 ± 0.001 vs 0.015 ± 0.004 vs 0.017 ± 0.006) (Fig. 7).

Coincubation with copper sulfate reduced cisplatin accumulation in mouse cochlea

The role of Ctr1 in cochlear uptake of cisplatin was determined by performing uptake experiments in the presence and absence of copper in freshly isolated mouse cochleae. The net uptake rate of cisplatin was 14.7 ± 1.11 (pmol/mg tissue · h). Under similar conditions when cisplatin was coincubated with copper sulfate (1 mM), a 40% reduction in platinum uptake was observed [control vs copper sulfate treated; 14.7 ± 1.11 vs 8.83 ± 1.05 pmol/mg tissue · h; $p < 0.01$]. These data suggest that cisplatin and copper enter the cochlea through a common uptake mechanism, underscoring the importance of Ctr1 in cochlear uptake of cisplatin.

In vivo proof-of-concept: intratympanic injection of copper sulfate protected against cisplatin ototoxicity

A dose–response curve was created in C57BL/6 mice through comparison of the elevation of ABR thresholds with progressively increasing doses of cisplatin. At a dose of 18 mg/kg, the click threshold shift was 11.5 ± 5 dB with no associated mortality. At 20 mg/kg, the mean ABR threshold shift was elevated to 26.2 ± 15.8 dB, with a mortality rate of 15% after 5 d. At a dose of 22 mg/kg, the ABR threshold increased by 40.5 ± 16.0 dB; however, the mortality rate increased to an unacceptable 30% after 5 d. Based on these results, 20 mg/kg was selected as an optimal ototoxic dose for cisplatin with acceptable mortality.

Similar studies were performed with intratympanic $CuSO_4$ or water without intraperitoneal cisplatin injection. No histological signs of tissue damage were observed with water-only injections and ABR thresholds were similar to normal mice. The doses >0.1 mg/kg showed a significant ABR threshold shift (>10 dB). Both 0.04 and 0.05 mg/kg doses showed a mean threshold shift of 5.33 ± 2.67 and 6.50 ± 1.50, respectively. The 0.025 mg/kg dose of copper showed only a marginal change in click ABR threshold (1 ± 1 dB) as well as the 8, 16, and 32 kHz frequencies. Morphologically, after exposure to 0.025 mg/kg copper sulfate, there was no damage to the hair cells along with other parts of cochlea (Fig. 8*a–d*), rendering it as a choice for the maximum tolerated nonototoxic dose of copper for the cisplatin ototoxicity protection experiment.

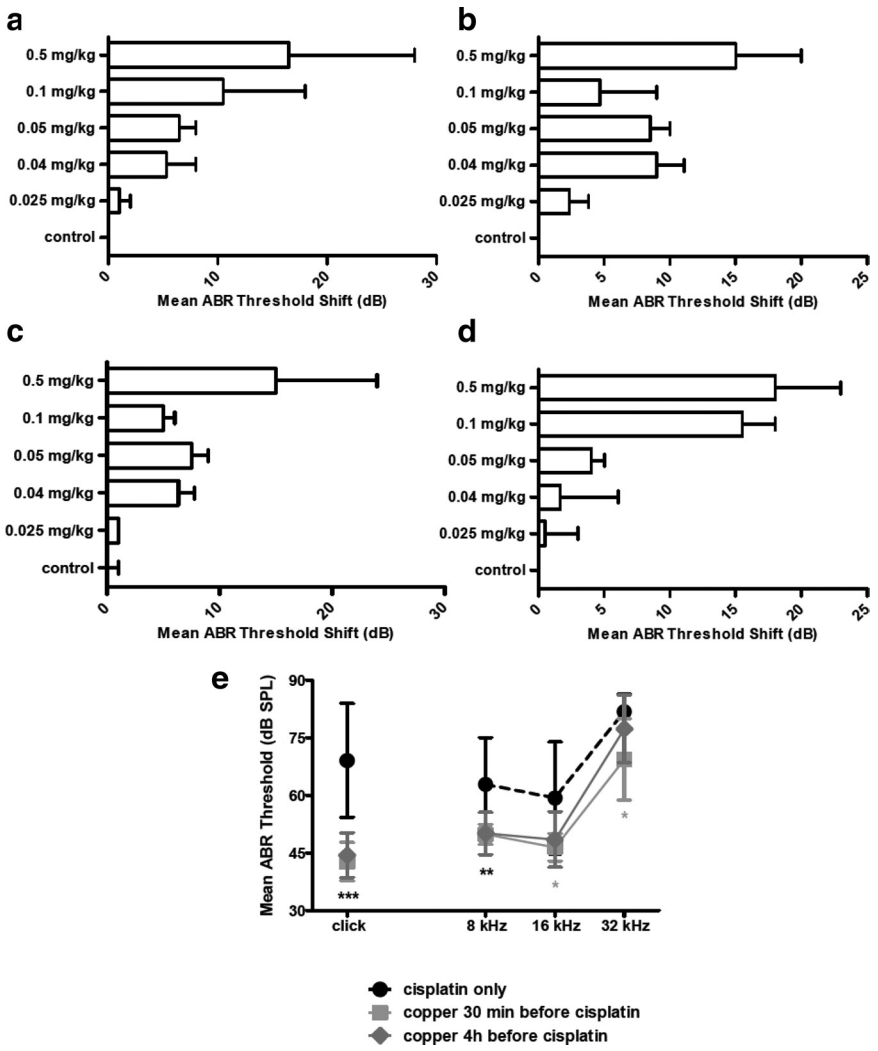


Figure 8. ***a–d***, Dose-dependent shifts in mean ABR thresholds for click (***a***) and at 8 kHz (***b***), 16 kHz (***c***), 32 kHz (***d***) frequency range 72 h after a single intratympanic delivery of copper sulfate. ***e***, Mean ABR thresholds for water (●) and copper sulfate (0.025 mg/kg; solid line)-treated ears 72 h after a single intraperitoneal administration of cisplatin (20 mg/kg; $n = 4$) in C57BL/6 mice. Statistically significant reductions in ABR thresholds were obtained at 8 kHz frequency (**$p < 0.01$) and for clicks (***$p < 0.0001$) when copper sulfate was administered 30 min (■) and 4 h (◆) before cisplatin treatment. Significant protection was observed at the 16 and 32 kHz frequency range in copper-pretreated ears 30 min before cisplatin administration (*$p < 0.05$). Data are represented as mean ± SEM.

The effect of intratympanic administration of copper (0.025 mg/kg) 30 min and 4 h before intraperitoneal cisplatin (20 mg/kg) on ABR threshold was next determined after 72 h (Fig. 8*e*). In animals pretreated with copper sulfate 30 min before cisplatin dosing, the cisplatin-induced changes in ABR thresholds were significantly less at click, 8, 16, and 32 kHz (9.75 ± 1.77 vs 36.1 ± 5.23 dB, 4.88 ± 0.91 vs 17.9 ± 4.29 dB, 1.50 ± 1.25 vs 14.4 ± 5.16 dB, and 21.38 ± 3.71 vs 33.9 ± 1.72 dB for click, 8, 16, and 32 kHz, respectively). In contrast, the mean ABR threshold changes in the copper sulfate pretreated ears 4 h before cisplatin were significant only for click and 8 kHz (11.4 ± 1.94 vs 36.1 ± 5.23 dB for click and 5.11 ± 1.84 vs 17.9 ± 4.29 dB for 9 kHz) (Fig. 8*e*). Similar results were obtained when ABRs were measured at 7 and 12 d. That is, copper continued to provide a protective effect against the deleterious effects of cisplatin on ABR thresholds.

The increase in auditory thresholds at the high frequencies was accompanied by extensive loss of outer hair cells in the basal and middle turns of the cochlea in cisplatin-treated animals along with intratympanic water (Fig. 9*a*,*b*). The basal region of the

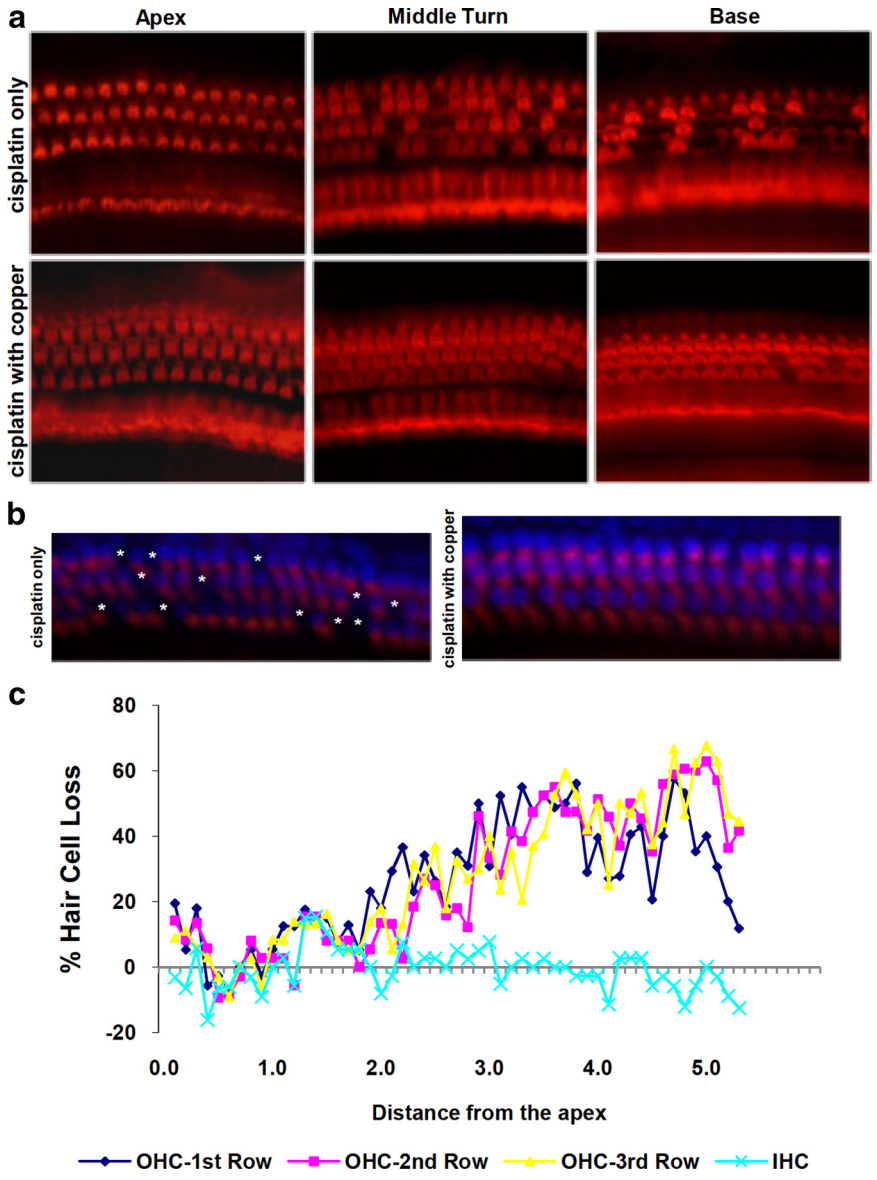


Figure 9. Copper sulfate prevents hair cell loss in cochlear explants of C56BL/6 mice treated with cisplatin. ***a***, Fluorescence microscopic images of phalloidin-stained organ of Corti explants showing one row of inner hair cells and three rows of outer hair cells. Intratympanic delivery of copper sulfate (0.025 mg/kg) protected against outer hair cell damage caused by intraperitoneal cisplatin (20 mg/kg) in basal and middle turns. ***b***, Immunostaining of whole-mount cochleae with a hair cell-specific marker, myosin VIIa, and the nuclear stain, DAPI (4′,6′-diamidino-2-phenylindole). Copper pretreatment protected against cisplatin-induced hair cell loss. The white asterisks indicate missing hair cells. ***c***, Hair cell cytocochleogram of the organ of Corti. Compared with copper-pretreated ears of mice, water-pretreated ears showed ~60% hair cell loss after cisplatin administration.

cochlea showed ~58% loss of outer hair cells and ~31% loss of outer hair cells in the middle turn. The outer hair cells in the apex region remained structurally unaffected as assessed by surface preparations. In contrast, the organ of Corti was well preserved in all turns of the ears pretreated with copper sulfate, as evident from its identical appearance to a physiologically normal organ of Corti. The percentage loss of outer hair cells was 9 and 5 in the basal and middle turn, respectively, whereas the apical turn did not exhibit hair cell damage. Toluidine staining of the permanent sections of cochleae treated with water or copper sulfate before administration of cisplatin also showed similar results (Fig. 10). Outer hair cell loss was detected only in water-treated ears but not in copper sulfate-pretreated ears. No significant damage was observed in stria vascularis and spiral ganglion cells in all treatment groups (supplemental Fig. S3, available at www.jneurosci.org as supplemental material).

Discussion

A true "chemoprotective" strategy for the attenuation of ototoxic effects of platinum would necessarily involve exploitation of differential molecular or pharmacokinetic mechanisms of cisplatin activity in ear cells versus tumor cells. Attenuation of untoward effects is often accompanied by reduction of desired antitumor effects. In lieu of extensive, unequivocal knowledge of such differences, rational formulation of chemoprotective strategies is, at best, difficult. The limited success of current attempted strategies such as specific counteraction of the effects of ROS and increased expression of HMG1 protein underscores the need for the development of rational chemoprotective or chemopreventive strategies against the ototoxic effects of platinum. The investigations in this study focus specifically on identifying and modulating the determinants for cisplatin uptake into cochlear tissue, the site of cisplatin ototoxicity.

To our knowledge, the cochlear expression of Ctr1, an influx transporter for platinums has not been studied. [While this manuscript was under review, Ciarimboli et al. (2010) published a study probing the role of OCT2 in cisplatin-induced ototoxicity and nephrotoxicity. Cimetidine, an OCT2 inhibitor, protected against cisplatin ototoxicity. Furthermore, OCT2 expression was found in the organ of Corti.] Our initial experiments using RT-PCR and qRT-PCR on mouse cochlear cDNA and HEI-OC1 cell line show high abundance of Ctr1. The cochlear tissue sample confirms the expression of OCT2. Although OCT2 was undetectable in the HEI-OC1 cell line, it might merely be attributable to loss of expression during the process of immortalization or compensation because of expression of another homologous transporter like OCT1. Western blot analysis of these transporters further corroborates the results from RT-PCR experiments. The localization of Ctr1 and OCT2 in mouse cochlea suggests their potential role in cisplatin ototoxicity. Major sites of cisplatin ototoxicity are in the inner ear, mainly the outer and inner hair cells followed by stria vascularis and spiral ganglion (Cardinaal et al., 2000). Immunohistochemistry in these studies indicates localization of Ctr1 in hair cells. Both transporters were detected at the secondary sites of cisplatin ototoxicity, namely stria vascularis and spiral ganglion. Our data suggest an important role of CTR1 in primary cisplatin ototoxicity to hair cells and that damage to secondary sites may be attributable to a combination of uptake processes mediated by CTR1 and OCT2.

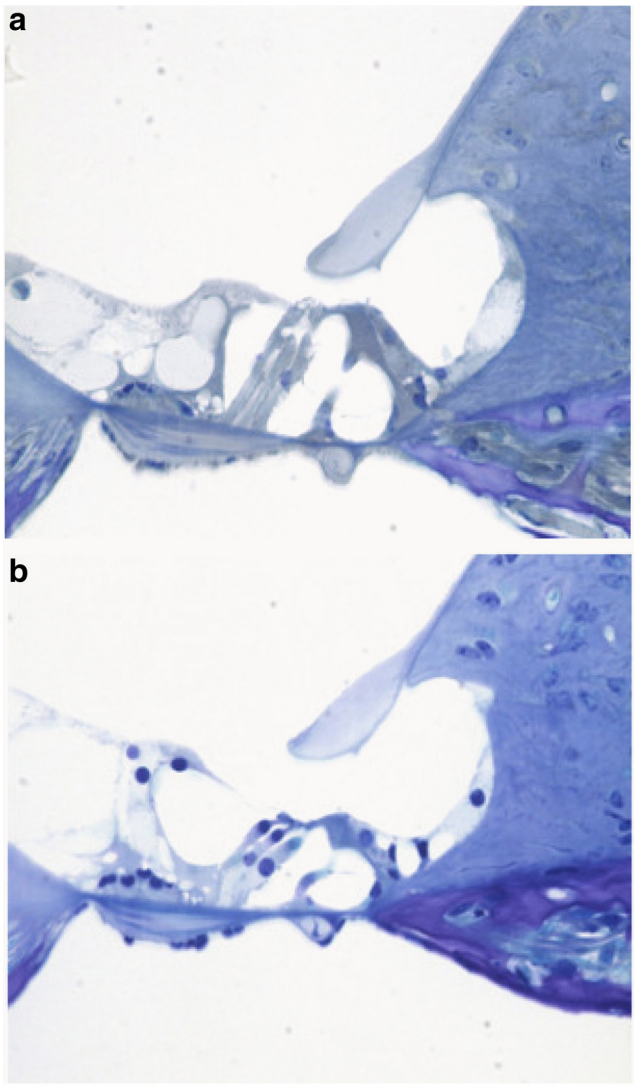


Figure 10. Cochlear histology of the C57BL/6 mice treated with cisplatin (***a***) and cisplatin plus copper sulfate (***b***). Loss of outer hair cells was evident in cisplatin-only treated mice, whereas copper sulfate clearly protected against this damage. There was no significant toxicity observed in the spiral ganglion and the stria vascularis in the case of both treatment groups.

Specific interaction of cisplatin with CTR1 was studied using CTR1-transfected HEK293 cells. HEK293 cells overexpressing CTR1 were more sensitive to cisplatin than the corresponding EV-transfected HEK cells, a phenomenon mirrored in an increased platinum accumulation and platinum-DNA adducts concentration in CTR1-transfected HEK293 cells after cisplatin treatment. These effects were inhibitable by $CuSO_4$, a specific CTR1 substrate. Treatment with copper resulted in reduced intracellular concentration of cisplatin and resulting cell death, suggesting similarities in the uptake mechanism via CTR1 used by both. The importance of CTR1 expression on cisplatin uptake and the resultant toxicity was thus established and suggested that the use of copper or another CTR1 inhibitor may be a viable chemopreventive strategy for cisplatin ototoxicity.

It is established that cisplatin causes hearing loss by inducing apoptosis (programmed cell death) of sensory hair cells. The major limitations in understanding the cellular and molecular mechanisms underlying drug-related ototoxicity have been limited availability of inner ear tissues and inability to model drug-induced ototoxic effects in laboratory animals (Kalinec et al., 2003). There are reports in the literature on the use of explants of cochlear and vestibular organs for such ototoxicity studies. Organotypic cultures are difficultly established, however, and the requirement of several explants for each experimental condition limits the scope of this technique. Consequently, an immortalized cell line derived from the organ of Corti, HEI-OC1, was used since it has previously been shown to express several proteins (such as myosin VIIa and Atoh1) suggestive of hair cell phenotype. This cell line is sensitive to ototoxic drugs such as cisplatin and gentamicin while being resistant to nonototoxic drugs, making it a simpler alternative for probing the role of CTR1 in cisplatin-induced ototoxicity (Kalinec et al., 2003). It has been previously used for evaluation of a variety of agents as potential chemoprotective entities against cisplatin-induced ototoxicity (So et al., 2008; Kim et al., 2009). However, these cells also express nestin, a protein expressed in the neonatal organ of Corti as well as the protein OCP2, which is expressed in supporting cells, demonstrating differences from mature hair cells (Ou et al., 2010). We also observed differences in the expression levels of transporters such as OCT1 and OCT2 between HEI-OC1 cells and cochleae harvested from mice. These data suggest that the cell lines can be used only as a starting point for drug toxicity studies and that follow-up *in vivo* studies must be performed to assess drug-induced ototoxicity. In this cochlear cell line, HEI-OC1, copper significantly attenuated the cytotoxicity and uptake of cisplatin. Copper sulfate itself did not significantly alter HEI-OC1 cells morphologically during the incubation period of the uptake study. Cochlear tissue uptake of cisplatin was also significantly reduced by copper. The effect of Ctr1 expression on cochlear uptake of cisplatin was demonstrated directly through the use of Ctr1-specific siRNA. The reduction in uptake of cisplatin in HEI-OC1 cells transfected with Ctr1-specific siRNA underscored the importance of Ctr1 in its uptake. These results highlight the significance of CtrR1-mediated cisplatin uptake in the cochlea.

To obtain an *in vivo* proof-of-concept for the contribution of CTR1 in cisplatin ototoxicity, we used $CuSO_4$ as a protective agent against cisplatin toxicity. Since the antitumor efficacy of cisplatin is dependent on its CTR1-mediated uptake in cancer cells, the protecting agent was delivered locally at the site of action through intratympanic injection and cisplatin was administered systemically. Pretreatment with $CuSO_4$ before cisplatin dosage was indeed protective at low, median, and high frequency ranges. The delivery of copper sulfate immediately before cisplatin injection produced a higher degree of protection from cisplatin ototoxicity than delivery 4 h before. Possibly, higher concentrations of copper were available locally to compete with cisplatin when copper was administered immediately before cisplatin treatment. Reduced uptake of cisplatin in presence of copper sulfate appeared to be the mechanism for this protection. In particular, the platinum concentration in the total cochlea after cisplatin administration was reduced by 20% in copper sulfate-pretreated ears when compared with water-treated ears ($p < 0.05$) (data not shown). Although statistically significant, a 20% reduction in platinum uptake by cochlea may be considered small; however, we are not able to measure specific cell-dependent uptake of platinum, for example, in outer hair cells. At the concentration tested, $CuSO_4$ failed to affect Ctr1 mRNA level (Holzer and Howell, 2006). It is important to note that, in addition to inhibition of CTR1, other mechanisms may be involved in the protective effects of copper on cisplatin ototoxicity. For example, copper (cuprous or cupric) may also itself enhance reducing mechanisms through incorporation into Cu^{+}/Cu^{2+} redox couple-containing

enzymes. Thus, the ototoprotection observed in this experiment might be, in part, attributable to reduction of ROS in cochlea and resulting apoptosis.

Cisplatin treatment has been shown to result in the reduction of glutathione levels and expression levels of antioxidant enzymes, mediated primarily through the resulting ROS (Rybak et al., 2007). Utilization of *N*-acetylcysteine, methionine, vitamin E, and ebselen have therefore been previously studied and have been shown to be effective in modulating cisplatin-induced ototoxicity in animal models. Unfortunately, clinical trials reported to date have been met with limited success, for example, that with amifostine, a phosphorylated aminothiol antioxidant. Other trials with different antioxidants are ongoing, so it is still possible that antioxidants may turn out to be useful modalities in otoprotection. Nevertheless, at this time, formulation of alternative strategies for cisplatin otoprotection is prudent. Because of inherent toxicity of copper sulfate, we were unable to administer it at higher doses, at which Ctr1 would be maximally inhibited. Copper(II) in solution (as $CuSO_4$) has been shown to affect hair cells in the low micromolar concentration range, in the lateral line neuromasts of zebrafish larvae (Hernández et al., 2006; Linbo et al., 2006). Therein, although micromolar concentrations of copper exhibited toxicity toward the hair cells, survival of the fish remained unaffected. This toxicity is dose dependent, with the damage to being completely reversible through cell regeneration below concentrations of 50 μM (Hernández et al., 2006). These results suggest that other less toxic or nontoxic CTR1 inhibitors for protection from cisplatin ototoxicity should be developed.

To summarize, the involvement of CTR1 in cochlear uptake and toxicity of cisplatin was unequivocally established through our *in vitro* experiments. Localization of Ctr1 and not of OCT2, in the hair cells of organ of Corti, makes it a primary candidate for mediating cisplatin ototoxicity. The final proof-of-principle *in vivo* experiment with copper sulfate protecting agent has provided a novel basis for understanding and designing otoprotective strategies for cisplatin therapy. Future studies involving less toxic analogs of copper sulfate or other inhibitors of CTR1 are warranted. Screening of small molecules for CTR1 inhibition and/or the use of silencer RNA approach should be considered.

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