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Evaluation of the *in vitro* otoprotective potential of 4-aminopyridine for cisplatin-induced ototoxicity

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Background: Cisplatin, an effective chemotherapy, is a cause of ototoxicity. It enters inner ear cells through Organic Cation Transporter 2 (OCT2). Recently, we identified another cochlear transporter, Multidrug and Toxin Extrusion Protein 1 (MATE1); it allows cisplatin efflux in renal cells. OCT2 and MATE1 appear to work together in eliminating renal toxins. 4-aminopyridine (4-AP), a known blocker of voltage-dependent K⁺ channels, blocks OCT2 but not MATE1 in the kidney. We hypothesize that 4-AP could prevent cisplatin from entering cochlear cells, without inhibiting the efflux of cisplatin that may have already entered. This could enhance clearance, mitigating ototoxicity.

Objective: Determine the otoprotective potential of 4-AP *in vitro* following cisplatin exposure.

Methods: Mice cochlear explants were treated with cisplatin (20 μM), with or without 4-AP (500 μM) for 24 h, evaluated for caspase 3/7 activity with Cell Event[®], visualized with confocal and phase contrast microscopy. Moreover, HeLa cells and cochlear explants were exposed to cisplatin (75 μM and 30 μM, respectively) with or without 4-AP in the explants and were evaluated for cisplatin-DNA adducts and cleaved caspase-3 immunohistochemistry.

Results: For cochlear explants exposed to cisplatin, Cell Event[®] staining occurred in the supporting cells, medial to the hair cells, and in the organ of Corti. No significant differences were found between cisplatin-exposed explants with or without 4-AP. In HeLa cells, consistent cellular staining for cisplatin-DNA adducts and cleaved caspase-3 was observed. However, this was not seen in cochlear explants, and there was no significant decrease in staining levels with 4-AP co-treatment.

Conclusions: In this *in vitro* study, no otoprotective potential of 4-AP against cisplatin-induced ototoxicity was observed. While 4-AP blocks OCT2, it did not decrease cisplatin-DNA adducts in cochlear cells. Further research into OCT2 and MATE1 transport mechanisms is needed.

KEYWORDS

4-Aminopyridine, cisplatin, MATE, MATE1, OCT2, organic cation transporter, ototoxicity, platinum

Introduction

Cisplatin is a widely used chemotherapeutic drug worldwide. It is known to cause ototoxicity, resulting in irreversible bilateral sensorineural hearing loss; it is also nephrotoxic (Karasawa and Steyger, 2015). The precise transporter involved in cisplatin entry into the cochlea is under investigation, but it is believed that cisplatin may enter through copper uptake protein 1 (Ctr1) (Schoeberl et al., 2022), and mainly through organic cation transporter 2 (OCT2) (Jeon et al., 2023). Once inside the cell, cisplatin binds to DNA, creating irreversible adducts that lead to cell death, primarily through apoptosis. Greater amounts of platinum-DNA adducts correlate with increased cytotoxicity (Breglio et al., 2017).

Recently, we have identified another cochlear transporter, the multidrug and toxin extrusion protein 1 (MATE1), which may be involved in cisplatin transport. We demonstrated that MATE1 is present in the cochlea; in hair cells, supporting cells, the stria vascularis, and the spiral ganglion neurons (Waissbluth et al., 2023). Interestingly, it has been observed that MATE1 increases the efflux of cisplatin from renal cells. It is now known that MATE1 works in conjunction with OCT2 to mediate the transcellular movement of its substrates, participating in cellular detoxification and reinforcing excretion mechanisms in the kidney (Motohashi and Inui, 2013). Also, it has been described that cisplatin-treated mice exhibit downregulation in the expression of MATE-1 and OCT-2 in renal tissue (Freitas-Lima et al., 2020).

Due to its potential role in cisplatin efflux, its pharmacogenetic role was studied in 206 patients with head and neck cancer who received cisplatin, and it was discovered that homozygous A/A variants for MATE1 (rs2289669) were significantly protected from developing ototoxicity; it would have a protective effect against ototoxicity (Teff et al., 2019). It is conceivable that cisplatin could exit inner ear cells through MATE1, thereby protecting cochlear cells from toxicity.

4-Aminopyridine (4-AP) is a voltage-dependent potassium channel blocker, approved by the FDA for multiple sclerosis (Kim, 2017). It can non-specifically block potassium channels, thereby prolonging the action potential, potentially improving neuronal conduction and peripheral motor neurological function. There are no reports of cochlear toxicity as a side effect of this medication (Belavic, 2010). Importantly, it was recently observed that 4-AP is a substrate and inhibitor of OCT2, but not of MATE1 in the kidney (Xiao et al., 2018). This suggests that it would be possible to administer 4-AP to inhibit cisplatin entry into the cochlea by blocking OCT2 without inhibiting MATE1, allowing cisplatin efflux from the cells and consequently reducing intracellular cisplatin content. Through this mechanism, it would be possible to protect inner ear cells from cisplatin toxicity.

We hypothesize that 4-AP could prevent cisplatin from entering cochlear cells, without inhibiting the efflux of cisplatin that may have already entered. This could enhance clearance, mitigating ototoxicity.

Objective

We hypothesized that 4-AP could prevent cisplatin entry into cochlear cells without inhibiting the efflux of cisplatin that may have already entered. This could improve elimination, mitigating ototoxicity. The aim of this study was to determine the otoprotective potential of 4-AP *in vitro* following cisplatin exposure.

Material and methods

Cell culture experiments

We first employed an *in vitro* model using HeLa cells. Immunohistochemistry was initially performed to assess the expression of OCT2 and MATE1. Subsequently, the cells were exposed to cisplatin 75 μ M [Pfizer (Perth) Pty Limited], and the formation of platinum-DNA adducts, and caspase-3 activation were evaluated as markers of DNA damage and apoptosis, respectively.

Independent biological experiments were performed on different culture dates (March 15, April 11, and April 25, 2023). For each experimental condition (control, 4-AP, cisplatin, and cisplatin + 4-AP), one coverslip per experiment was analyzed ($n = 3$ per condition). From each coverslip, 3–4 randomly selected microscopic fields were acquired. Quantitative measurements from individual fields were averaged to obtain a single value per experiment. Statistical analyses were performed considering the independent biological experiment as the experimental unit. HeLa cells were cultured as in Abbott et al. (2023). Briefly, cells were cultured in Minimum Essential Medium (Gibco™ Catalog number 41500034, Thermo Fisher Scientific) supplemented with 5% fetal bovine serum (Sigma-Aldrich, catalog number A7906) and antibiotic/antimycotic solution (100x) (Capricorn Scientific, catalog number AAS-B) containing: amphotericin B 25 mg/L, NaCl 9,000 mg/L, penicillin G sodium 10 Units/L, sodium deoxycholate 205 mg/L, streptomycin sulfate 10,000 mg/L at a final concentration of 10 ml/L. Experiments were conducted when cell confluence reached approximately 75%. Cells were then washed with PBS1x and fixed with 4% paraformaldehyde (in PBS) for 30 min. Immunofluorescence was initially performed to detect the transporters OCT2 and MATE1, using anti-OCT2 polyclonal antibody (OCT21-A, Alpha Diagnostic International Inc.) and anti-MATE1 polyclonal antibody (ab224440, Abcam), respectively. Primary antibodies were diluted in blocking solution at a ratio of 1:500 (1:200 for MABE416), and samples were incubated in a humid chamber for 3 h at room temperature or, alternatively, overnight at 4 °C. In a separate experiment, HeLa cells were treated with 75 μ M cisplatin. Subsequently, immunostaining was performed to detect DNA adducts formed by cisplatin [Anti-Cisplatin DNA Adducts Antibody, clone ICR4 (MABE416, Merck)] and cleaved caspase-3 [Cleaved Caspase-3 (Asp175) Antibody #9661, Cell Signaling Technology] to assess cell death. Percentage of cleaved caspase-3 positive cells were also calculated. Secondary antibodies: Donkey anti-Rat IgG (H + L) highly cross-adsorbed

secondary antibody, Alexa Fluor™ 488, (catalog number A-21208, Thermo Fisher Scientific) Goat anti-Rabbit IgG (H + L) cross-adsorbed secondary antibody, Alexa Fluor™ 568, (catalog number A11011, Thermo Fisher Scientific). Secondary antibodies were used at dilutions ranging from 1:200 (Anti-Rat only) to 1:500 in blocking solution, with incubation for 45 min at room temperature. For image processing of HeLa cells, confocal microscopy images were acquired at 40× magnification, and caspase-positive cells were counted vs. cells showing only DAPI staining. This analysis was limited to cell counting.

Cochlear explant experiments

Cochlear explants were prepared as in [Waissbluth et al. \(2023\)](#). Cochleae were dissected from C57BL/6 postnatal day 5 to 7 (P5–P7) mice, which were obtained from the central animal facility of the Universidad de Valparaiso. All procedures were approved by the internal ethics committee (certificate of the bioethics committee of the Faculty of Sciences of the University of Valparaiso ID CBC562022). All procedures were conducted following the NIH guide for the care and use of laboratory animals. The mice were sacrificed by decapitation. Both cochleae were obtained by skull dissection and brain removal. The cochleae were delicately extracted. The lateral wall, including the stria vascularis, was removed to allow clear visualization of the organ of Corti from the apical side. The dissection was performed using a bath solution made with (in mM) 138 NaCl, 5 KCl, 6 Glucose and 10 HEPES, pH 7.4, and previously filtered and stored at 4 °C. The tissue is fixed in the basal region and the membrane labyrinth is extended radially out and split from the central modiolum. The sensory epithelium is then separated from the stria vascularis and spiral ligament and transferred to a coverslip previously treated with Geltrex (Gibco A1569601, Life Technologies). Once that tissue is attached to the substrate, the coverslip is covered with culture medium: Dulbecco's Modified Eagle Medium (DMEM) with GlutaMAX™ (ThermoFisher Scientific, #10566016), with ampicillin (3µg/mL) and supplemented with 5% fetal bovine serum (Capricorn Scientific, catalog number FBS-HI-12A), and 5% horse serum (Gibco, catalog number 16050-122), and incubated for 24 h at 37 °C with 5% CO₂ and 95% humidity. We have previously described the presence and localization of OCT2 and MATE1 in a prior study, hence this experiment was not repeated ([Waissbluth et al., 2023](#)).

Ototoxicity studies in cochlear explants

4-Aminopyridine treatments. The explants were exposed to various concentrations of 4-AP (Sigma-Aldrich, catalog number 275875), 0.1 mM, 0.5 mM, and 1 mM for 72 h to assess potential ototoxicity as there is no prior reported literature about the potential ototoxic effects of 4-AP. After treatment, the cultures were evaluated using DAPI for nucleic acid staining (Sigma-Aldrich, catalog number D9542) to assess cell membrane permeability, CellEvent™ staining to detect activated caspase-3/7 in live cells

(Invitrogen, Thermo Fisher Scientific, catalog number C10423), and phalloidin staining to examine the structural integrity of the hair cell stereocilia. (Groups: cisplatin, control, 4-AP, cisplatin + 4-AP/4-7 cochleae per group). For all experiments, the experimental unit (n) was defined as one independent cochlear explant (one cochlea from different animals). A total of 15 cochleae obtained from three independent culture dates (December 23, 2022; January 18, 2023; and February 27, 2023) were analyzed. Explants were distributed as follows: cisplatin ($n = 4$), 4-AP ($n = 3$), cisplatin + 4-AP ($n = 4$), and control ($n = 4$).

For apoptosis assessment, the CellEvent™ assay was performed using the CellEvent™ caspase-3/7 Detection Reagent and following the manufacturer's instructions. This assay detects activated caspase-3/7 in live cells. The cochlear explants were evaluated at 3-, 6-, 12-, and 24-h post-exposure using a contrast fluorescence microscope. Fluorescence intensity in the hair cells was assessed, with higher fluorescence indicating greater activation of caspase-3/7. To determine the fluorescence intensity of hair cells in each organ of Corti explant, images were acquired at 3, 6, 12, and 24 h post-treatment. In each image, the area corresponding to individual hair cells was manually delineated using ImageJ Fiji software. Fluorescence intensity was obtained as the mean fluorescence value of each region of interest (ROI). Background signal was subtracted from these values and calculated as the average fluorescence of three signal-free areas selected within the same image. Background-corrected fluorescence values were subsequently plotted and analyzed using GraphPad Prism 8.0, and data were expressed as mean ± standard error of the mean (SEM).

For image processing in the CellEvent™ experiments, images were acquired using a Nikon Eclipse Ti epifluorescence microscope equipped with a Hamamatsu ORCA-Flash2.8 C11440 digital camera, and NIS-Elements AR 4.30.01 (64-bit) software was used for image acquisition. Images were captured with an exposure time of 500 ms. After completion of the CellEvent™ experiments, samples were fixed and subsequently analyzed by confocal microscopy. Cilia were labeled with red phalloidin. Details of microscopy and imaging can be found in [Supplementary material](#).

For the DAPI and Phalloidin (Invitrogen, Thermo Fisher Scientific, catalog number R415) staining, cochlear explants were fixed with 4% paraformaldehyde (in PBS 1x) for 30 min at room temperature and subsequently permeabilized using cold 70% ethanol or a solution of PBS 1x with 1% Triton X-100 for 20 min. Phalloidin staining was performed for 30 min at room temperature, following the manufacturer's instructions. After appropriate washing steps, DAPI (0.5µg/ml) was applied for 3 min to label cell nuclei. Finally, explants were mounted using Fluoromount-G™ medium and analyzed by confocal microscopy.

Microscopy details

Images were acquired by confocal microscopy using a Nikon C1 system (EZ-C1 software, Silver Version 3.91) equipped with a

standard detector and a Plan Fluor 40×/1.30 NA oil-immersion objective. For organ of Corti explants, Z-stacks were acquired with 1 μm optical sections to encompass the full thickness of the sensory epithelium and preserve the three-dimensional organization of the tissue. In contrast, HeLa cells were imaged in a single optical plane corresponding to the cellular equatorial plane, without Z-stack acquisition, due to their monolayer growth and two-dimensional morphology, which allows adequate visualization and quantification in a single optical section. Images were captured at a resolution of 1,024 × 1,024 pixels, with a field of view of 318.3 μm and an XY pixel size of 0.31 μm. However, the images presented in the explant figures correspond to regions of interest cropped from the original acquisitions. Adjustments were made solely for presentation purposes and did not alter quantitative information or spatial calibration. Additionally, the pinhole was set to 60 μm (medium setting) for both organ of Corti explants and HeLa cells. Lasers at 408 nm (13%), 488 nm (16%), and 543 nm (16%) were used in both experimental models. Images were acquired using three-pass scanning, with detector gain and

laser power kept constant across experimental conditions to enable quantitative comparisons.

Cisplatin ototoxicity

Cochlear explants were then treated with cisplatin 20 μM, with or without 4-AP, for 24 h. Following treatment, the presence of platinum–DNA adducts was evaluated, along with CellEvent® staining for activated caspase-3/7. The 4-AP dose was selected based on the previously described experiment (0.5 mM), and in the explants exposed to both 4-AP and cisplatin, the explants were pre-treated with 4-AP for 1 h prior to adding cisplatin.

Statistical analysis

All quantitative data were analyzed using GraphPad Prism 8.0 (GraphPad Software, San Diego, CA, USA). Data are

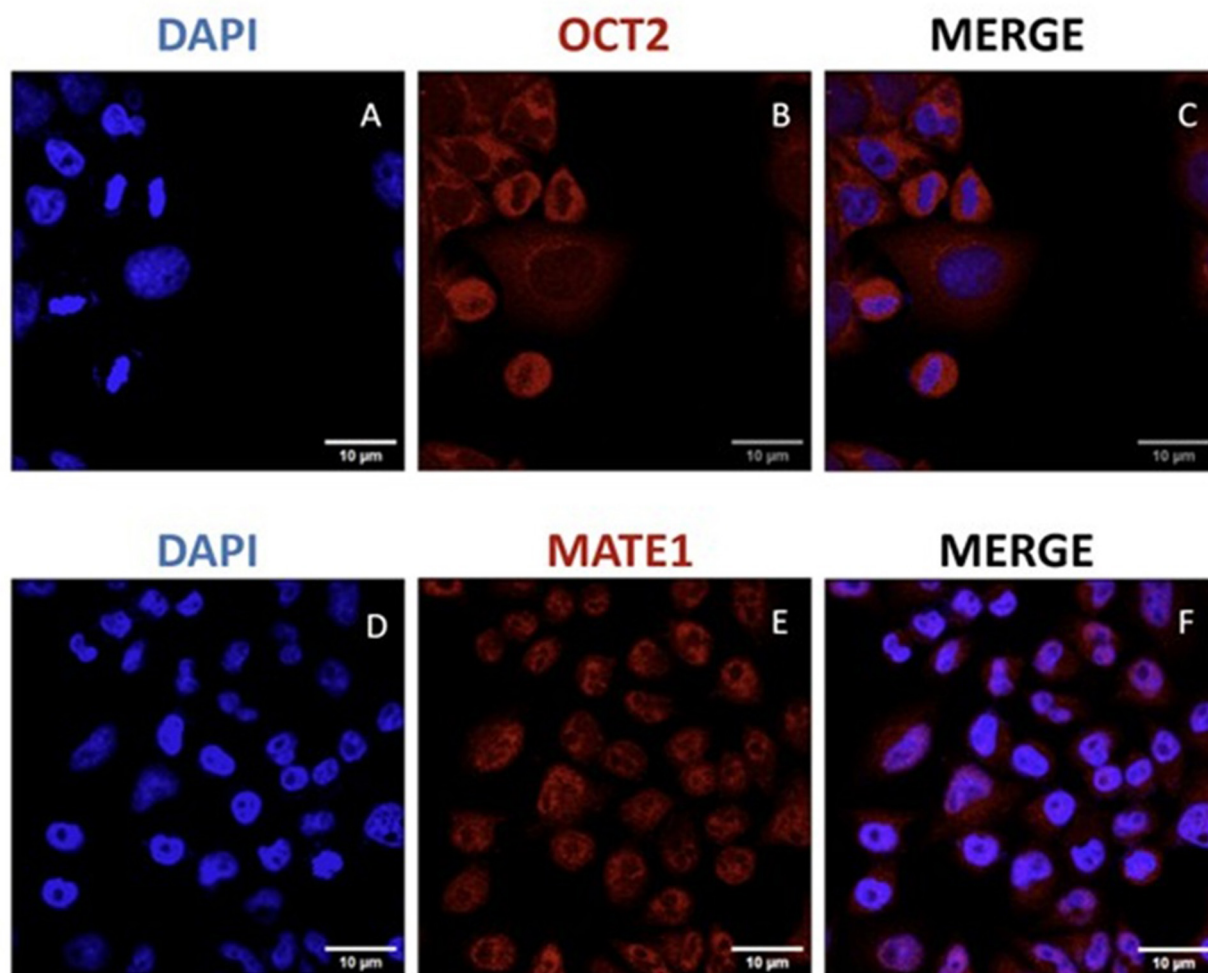
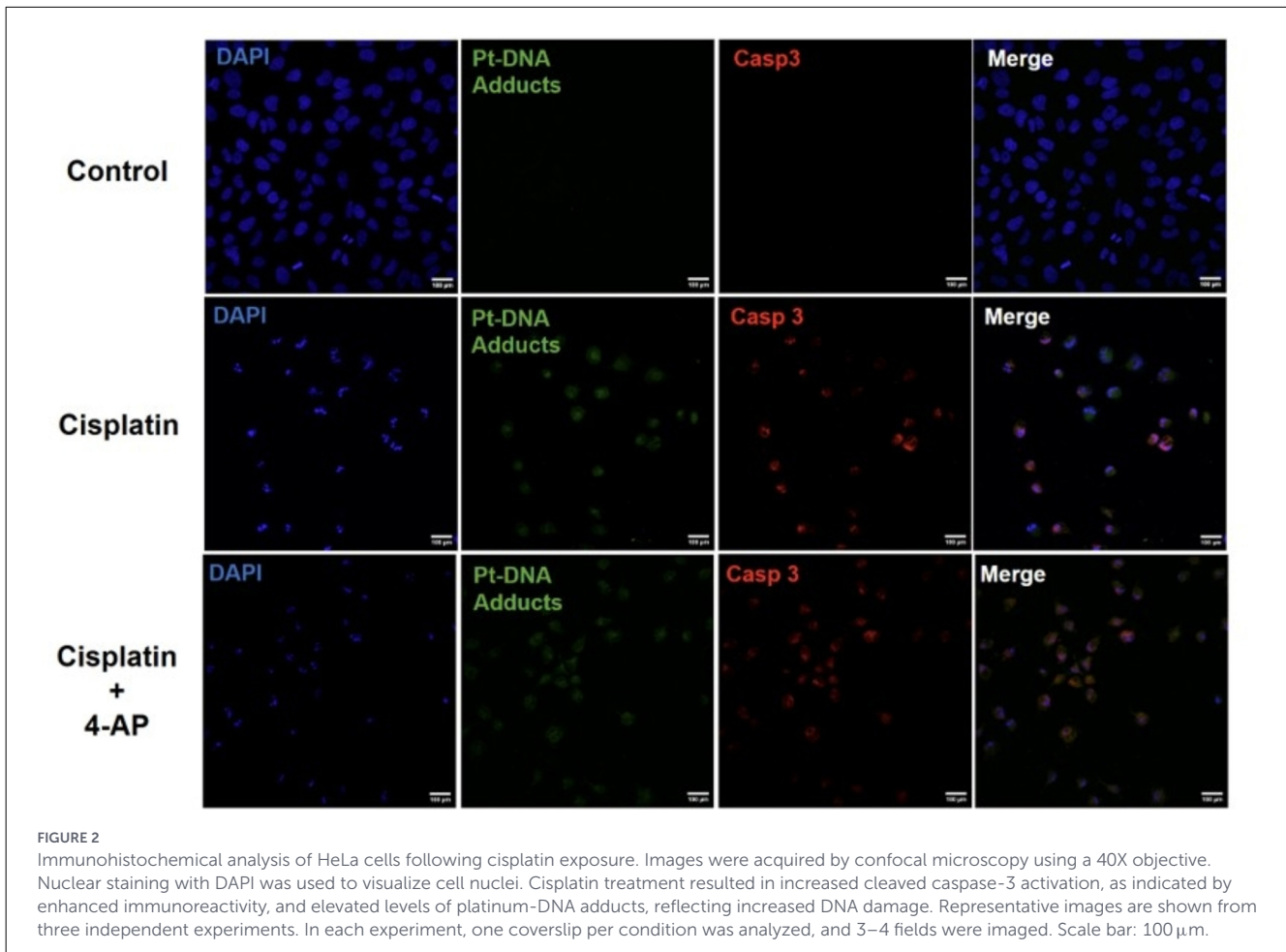


FIGURE 1
 Immunohistochemical staining of cultured HeLa cells. 40X. DAPI staining was used to visualize cell nuclei (A, D). Positive immunoreactivity for the transporters OCT2 (B) and MATE1. (E) was observed, indicating their expression in the cultured cells. (C) is a merge of (A) and (B), and (F) is a merge of (D) and (E).



presented as Mean ± Standard Error of the Mean (SEM). A *P* value < 0.05 was considered statistically significant. For the experiments performed with HeLa cells, data normality was assessed using the Shapiro–Wilk test, which indicated that the data are normally distributed. Accordingly, a one-way analysis of variance (ANOVA) was performed to compare group means. Homogeneity of variances was evaluated using the Brown–Forsythe test, which revealed no significant differences among group variances (*P* = 0.0758), confirming that the assumptions of the model were met. Multiple comparisons between groups were conducted using Tukey’s *post hoc* test. For the Cell Event™ experiments in cochlear explants, the assumption of normality was not met; differences among groups were analyzed using the non-parametric Kruskal–Wallis test (with Dunn’s *post hoc* correction). Data distribution was assessed using the Anderson–Darling, D’Agostino–Pearson, Shapiro–Wilk, and Kolmogorov–Smirnov normality tests. Independent explants were analyzed for each experimental group (control *n* = 4; cisplatin *n* = 4; cisplatin + 4-AP *n* = 4; 4-AP *n* = 3). Within each explant, multiple ROIs corresponding to individual cells were defined for intensity analysis. The number of biological replicates corresponds to independent cochlear explants from different animals or independent cell culture experiments, as specified in the figure legends.

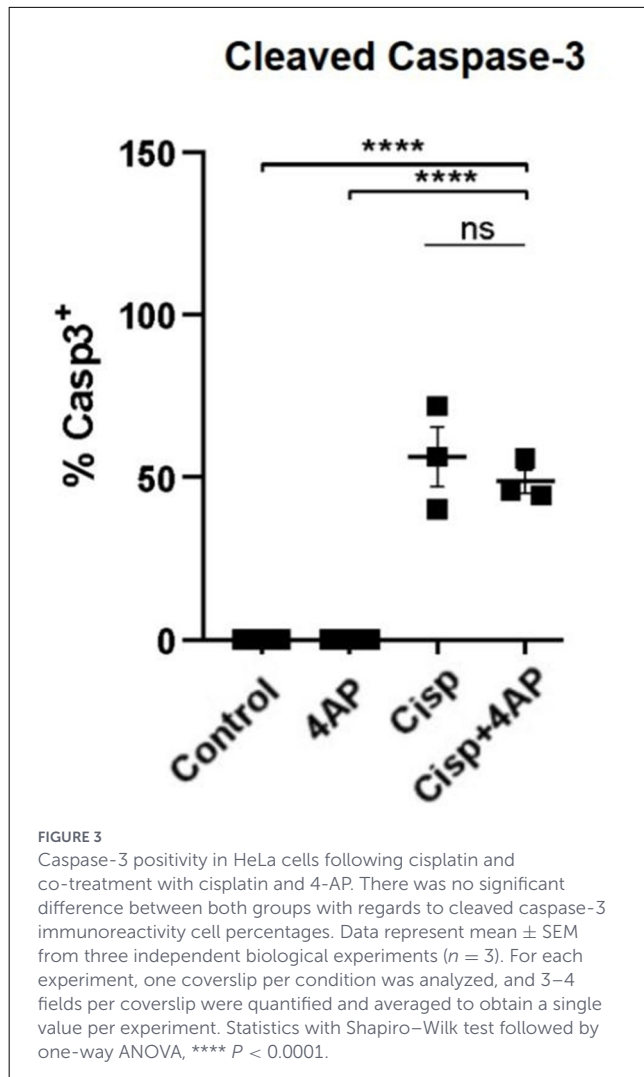
Results

HeLa cells express OCT2 and MATE1

HeLa cells were used as an *in vitro* model as they allow observation of cellular responses like apoptosis, DNA damage, and transporter expression (e.g., OCT2, MATE1) in a controlled environment. DAPI staining (Figures 1A, D) was used to visualize the cell nuclei. As we can see in Figures 1B, E, these cells express both OCT2 and MATE1 (merge Figures 1C, F).

Cisplatin exposure increases cleaved caspase-3 along with DNA adducts in HeLa cells

Once it was determined that HeLa cells expressed the transporters of interest, cell cultures were then exposed to cisplatin. The aim of this experiment was to assess whether cells exposed to cisplatin formed platinum–DNA adducts, and if these same cells expressed cleaved caspase-3 as a marker of apoptosis. The cisplatin-exposed cells show immunoreactivity for cleaved caspase-3 (Figure 2), and the same cells also exhibit immunoreactivity for



DNA adducts (Figure 2). No staining was observed in the control samples. Percentage of cleaved caspase-3-positive cells did not reveal any significant difference between the cells treated with cisplatin alone or with 4-AP c-treatment (Figure 3).

In this *in vitro* model, cultured cells express both OCT2 and MATE1 transporters; upon exposure to cisplatin, they exhibit cleaved caspase-3 activation, and the formation of DNA adducts.

Evaluation of the safety of 4-aminopyridine in cochlear explants

For the second part, we exposed cochlear explants to varying doses of 4-AP, using DAPI (Figures 4A–D) as well as Phalloidin staining to assess nuclear and stereocilia integrity, respectively. These explants were assessed for activated caspase-3/7 immunoreactivity with the CellEvent™ assay (Figures 4E–H). Immunoreactivity for activated caspase-3/7 increased as the concentration of 4-AP was increased. An important difference in staining was observed with 1mM of 4-AP (Figure 4H), indicating enhanced apoptotic activity. Phalloidin staining revealed

(Figures 4I–L) progressive disruption of stereocilia structure at higher 4-AP concentrations, with notable loss of structural integrity at 1 mM (Figure 4L).

Starting at a concentration of 1 mM 4-AP, cellular permeability is altered, as indicated by increased CellEvent™ fluorescence, and the structure of the stereocilia is disrupted (merge Figures 4M–P).

Evaluation of the potential otoprotective effect of 4-AP against cisplatin induced toxicity in cochlear explants

After determining a safe dose of 4-AP (0.5 mM), cochlear explants were exposed to cisplatin with and without 4-AP. The explants were first assessed for cisplatin–DNA adduct formation. Immunostaining revealed the presence of cisplatin-modified DNA in hair cells following cisplatin exposure (Figure 5). Although adducts were also detectable in explants co-treated with cisplatin and 4-AP (Figure 5), their staining intensity was reduced compared to those treated with cisplatin alone, indicating decreased DNA modification.

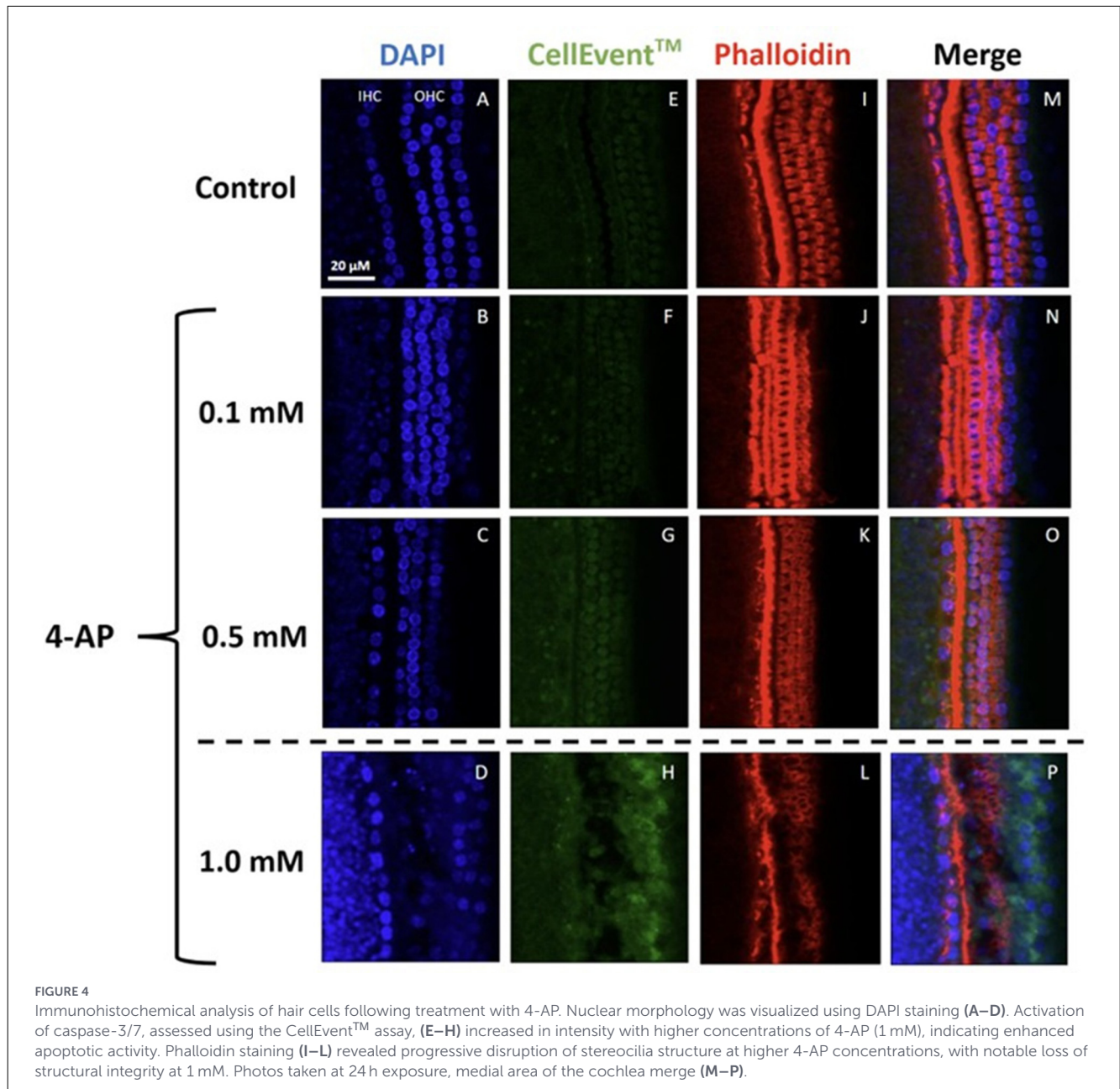
The explants were also evaluated for activated caspase-3/7 activity with the CellEvent™ assay. Staining was observed for inner and outer hair cells as well as the supporting cells and was significantly increased with exposure to cisplatin (P < 0.0001). There were no significant differences between explants treated with cisplatin alone and those co-treated with cisplatin and 4-AP (Figure 6). We did observe a trend in the supporting cells, for decreased staining for co-treated cells vs. cisplatin alone. Interestingly, a very slight increase in staining was observed in the group receiving 4-AP alone vs. control, in the supporting cells, not hair cells. Because of these results, we decided to perform cleaved caspase 3 immunohistochemistry as well.

Cleaved caspase-3 immunostaining was observed in cochlear explants following cisplatin exposure (Figure 7). A slight reduction in caspase-3 immunoreactivity was observed in explants co-treated with cisplatin and 4-AP (Figure 7).

Discussion

Cisplatin-induced ototoxicity remains one of the most debilitating adverse effects of this widely used chemotherapeutic agent. Permanent, bilateral sensorineural hearing loss is reported in as many as 80 % of patients (Frisina et al., 2016), and auditory thresholds may deteriorate or new deficits may surface months to years after therapy has concluded (Waissbluth et al., 2018). It is known that hearing loss can lead to academic learning and psychosocial difficulties in children (Gurney et al., 2007; Yancey et al., 2012). Also, in adults hearing loss can lead to depression and social isolation (Cosh et al., 2019), and is considered a risk factor for dementia (Thomson et al., 2017). It is, therefore, of great significance finding a preventive measure or treatment for the ototoxicity resulting from cisplatin chemotherapy.

The main inner ear targets for cisplatin include hair cells, the marginal cells of the stria vascularis and the spiral ligament fibrocytes (van Ruijven et al., 2005). Various studies have



demonstrated the presence of OCT2 in the inner ear, yet the localization in the cochlea has been debated. It has been previously detected in hair cells and the stria vascularis of the mouse cochlea (Ciarimboli et al., 2010). More et al. found it to be expressed in the spiral ganglion neurons and the stria vascularis of the mouse cochlea, but absent in the hair cells (More et al., 2010). Hellberg et al., describe similar results, reporting that OCT2 was observed in the supporting cells and type I spiral ganglion cells in the cochlea of rats, guinea pigs, and a pig (Hellberg et al., 2015). We also found it to be in the stria vascularis, spiral ganglion neurons and pillar cells (Waissbluth et al., 2023). In the kidney, OCT2 is highly expressed in the basolateral membrane of proximal tubules and is considered the main transporter mediating cisplatin accumulation (Okuda et al., 1996). Because intracellular cisplatin burden correlates directly with cytotoxicity, delineating the role of transporters such as

OCT2 is central to understanding, and ultimately preventing, both nephrotoxicity and ototoxicity associated with this drug.

Various transporters have been considered for potential efflux mechanisms of cisplatin in cochlear cells including ATP7A and ATP7B, P-type copper-transporting ATPases, which are copper pumps responsible for cisplatin sequestration and efflux, both found in the organ of Corti, spiral ganglion cells and stria vascularis (Ding et al., 2011). Elevated expressions of ATP7A and ATP7B have been associated with cisplatin resistance for various types of cancers; this is not clearly understood (Zhu et al., 2017). Recently, there has been evidence that the multidrug and toxin extrusion protein 1 (SLC47A1), MATE1, increases the efflux of cisplatin from renal cells, and is involved in cisplatin-induced nephrotoxicity. There is some evidence of an interplay between the uptake of cisplatin by OCT2 and efflux by MATE1 in the kidney,

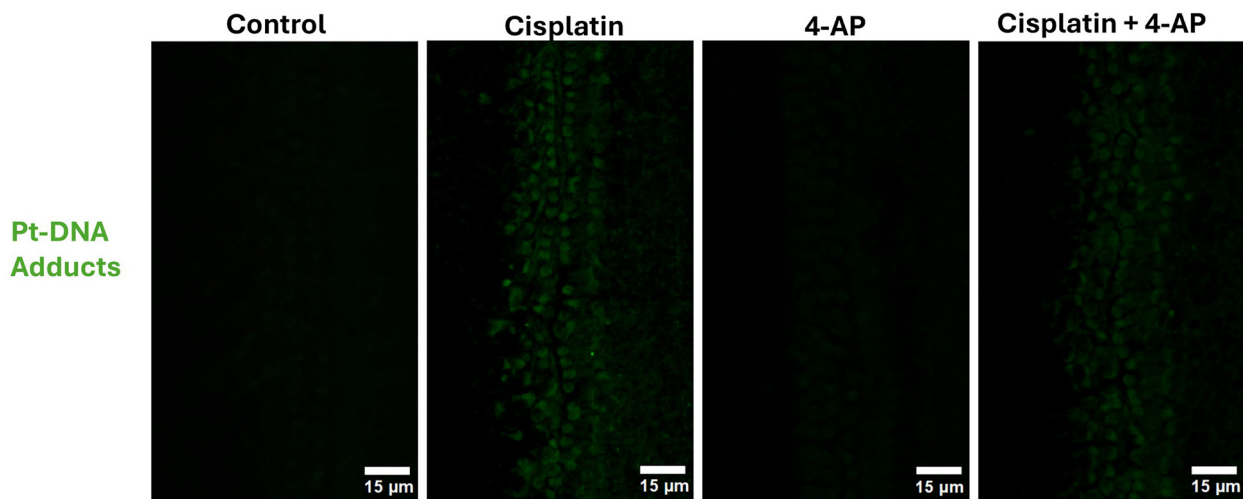


FIGURE 5

Cochlear explants exposed to cisplatin and 4-AP. Immunohistochemistry: Cisplatin-modified DNA is detectable in hair cells following cisplatin exposure. Cells treated with both cisplatin and 4-AP also exhibit staining for cisplatin-DNA adducts; however, the signal intensity is reduced compared to cisplatin-only treatment, indicating decreased DNA modification. The experimental unit (*n*) was defined as one independent cochlear explant (one cochlea). A total of 15 cochleae obtained from three independent culture dates were analyzed and distributed as follows: control (*n* = 4), cisplatin (*n* = 4), 4-AP (*n* = 3), and cisplatin + 4-AP (*n* = 4). Scale bar: 15 μm.

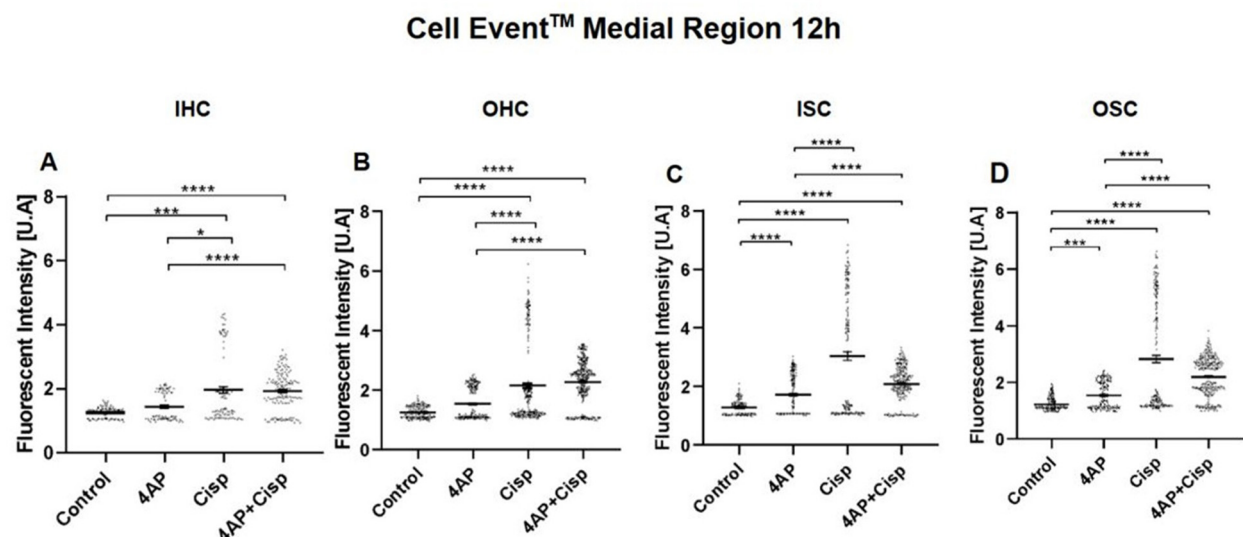


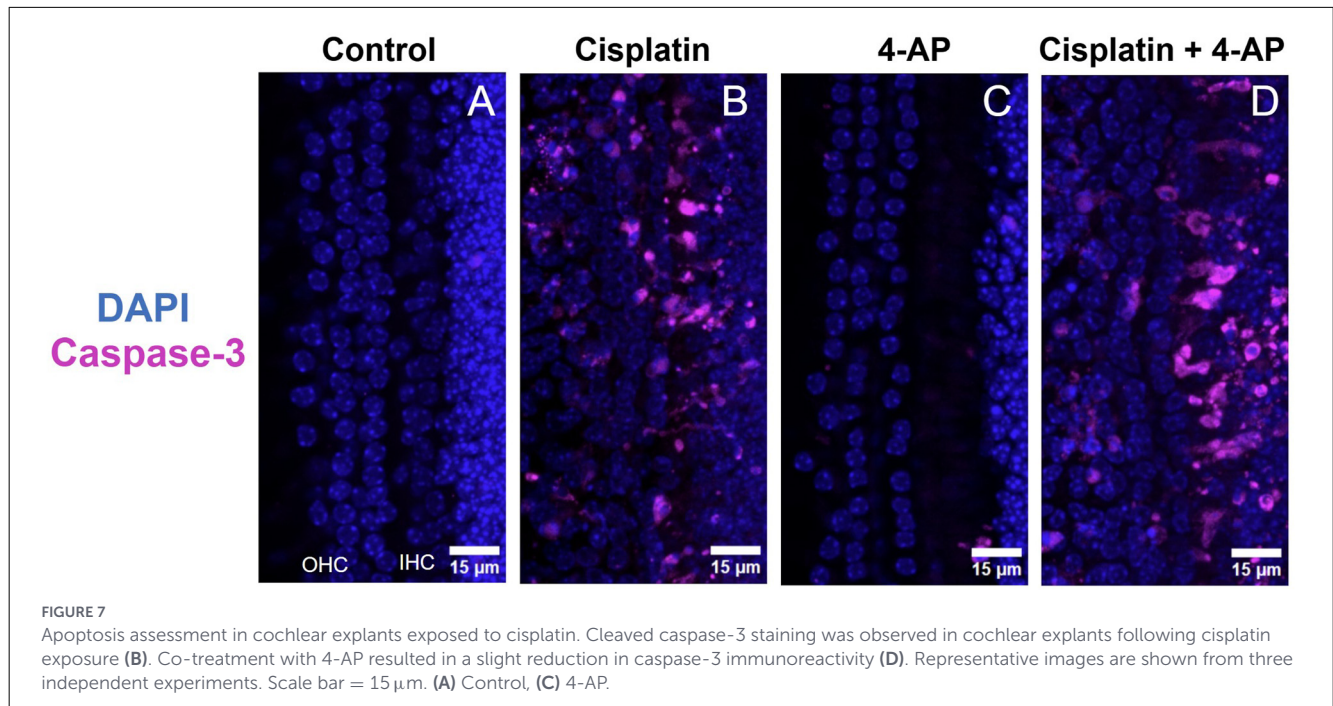
FIGURE 6

CellEvent® staining in cochlear explants exposed to cisplatin. CellEvent® staining was observed within the organ of Corti. Mean ± SEM. IHC, inner hair cells; OHC, outer hair cells; ISC, inner supporting cells; OSC, outer supporting cells. For each explant, all identifiable cells of each cell type were analyzed by individual ROI selection and background subtraction. The intensity values corresponding to the analyzed cells were included in the statistical analysis. No significant differences were detected between explants treated with cisplatin alone and those co-treated with cisplatin and 4-AP for any cell type (A–D). We see a trend of decreased staining in the supporting cells that were co-treated. Explants were distributed as follows: cisplatin (*n* = 4), 4-AP (*n* = 3), cisplatin + 4-AP (*n* = 4), and control (*n* = 4). Statistics with Kruskal Wallis test. **** *P* < 0.0001, *** *P* = 0.002, * *P* = 0.0128.

and pharmacological inhibition of MATE1 potentiates cisplatin nephrotoxicity (Nakamura et al., 2010). Importantly, MATE1 has recently been identified in the inner ear; it was observed in the stria vascularis, the spiral ganglion neurons and the organ of Corti (Waissbluth et al., 2023).

There is evidence that substrates or inhibitors of OCT2 and MATE1 can overlap, with different relative inhibition rates

(Ivanyuk et al., 2017; Chen et al., 2017). Yet, there is also evidence of selective inhibition for either transporter as well (Hibma et al., 2016; Kido et al., 2011). Of importance, recently, it was observed that Fampridine (also known as 4-AP) is a substrate and inhibitor of OCT2, but not for MATE1 (Xiao et al., 2018). Xiao et al. describe that the uptake of Fampridine by Hek293 cells is mediated by OCT2, and that uptake was OCT2 specific



and time-dependent. They showed that Fampridine inhibited the uptake of another OCT2 substrate (metformin). They also report that in MATE1 expressing cells, they did not observe any significant uptake of Fampridine, and that inhibiting MATE1 with a MATE1 prototypical inhibitor (cimetidine), did not alter the uptake (Xiao et al., 2018). Cisplatin is a substrate for OCT2 and MATE1 (Motohashi and Inui, 2013), and *Mate1*^{-/-} mice are more susceptible to cisplatin-induced nephrotoxicity as compared to the wild-type mice; they exhibit greater renal accumulation of platinum, because the cisplatin efflux mechanism is blocked (Nakamura et al., 2010). It has also been shown that ondansetron, a MATE inhibitor, intensified the nephrotoxic effect of cisplatin (Li et al., 2013).

In a hippocampal culture study, following 24 h of incubation with 4-AP, a decrease in mitochondrial metabolism was observed, along with a clear dose-dependent reduction in cell viability starting from a concentration of 1 mM (Del Pino et al., 2015). It could be that this dose is toxic in various cell types, which would be consistent with our cochlear explant results. As a result, we chose 0.5 mM of 4-AP for the cochlea explant experiments. In the explants exposed to cisplatin, CellEvent[®] staining was observed in supporting cells, medial to the hair cells, and in the organ of Corti. Immunostaining was evident in the hair cells; however, the addition of 4-AP did not result in a significant difference in staining compared to the cisplatin-treated cells. In fact, an increase in staining was observed in the group receiving both treatments. Regarding caspase-3, we observe staining for cleaved caspase-3 in cochlear explants exposed to cisplatin. However, upon addition of 4-AP, a slight decrease in staining was observed. No significant differences were found between the cochlear explants exposed to cisplatin with and without 4-AP. In the HeLa cells, consistent staining was observed for platinum-DNA adducts and caspase-3. This was also observed in cochlear explants. However, there was no

decrease in staining levels in the explants with co-administration of 4-AP.

A limitation of this study is that OCT2 functional inhibition by 4-AP was not directly quantified using a transporter activity assay (e.g., uptake of a fluorescent OCT2 substrate). Therefore, although the concentration of 4-AP used is reported to interact with OCT2, we cannot determine the extent of transporter inhibition achieved in cochlear cells under our experimental conditions. Thus, while the concentration of 4-AP employed is known to interact with OCT2 (in our study, we used 0.5 mM 4-AP—a concentration substantially above the reported IC₅₀ for OCT2 interaction—and included a 1-h pre-incubation before cisplatin exposure, therefore, OCT2 interaction by 4-AP would be expected under our conditions), the degree of transporter inhibition in cochlear explants remains inferential, and incomplete pharmacological blockade could contribute to the absence of observed otoprotection.

Although, genetic deletion of OCT2 has been shown to reduce cisplatin toxicity in renal tissue (Franke et al., 2010; Filipski et al., 2009) and has been proposed as a protective mechanism in other systems. Cochlear cells express multiple transport systems involved in cisplatin handling (CTR1, OCT family members, ATP7A/B, and MATE1) (Waissbluth et al., 2023, 2022). OCT2 contribution in the cochlea may be smaller or redundant compared with kidney proximal tubules (Yu et al., 2025), so partial pharmacological competition may be insufficient to alter net cisplatin accumulation.

Conclusion

This study explored the potential otoprotective effect of 4-AP against cisplatin-induced cochlear toxicity. Despite its selective inhibition of OCT2 and sparing of MATE1 observed in renal

models, 4-AP did not confer measurable protection in cochlear explants. Cisplatin exposure continued to elicit apoptosis and DNA adduct formation, with no significant reduction following 4-AP co-treatment. Moreover, higher concentrations of 4-AP were associated with cellular toxicity, suggesting a narrow margin for safe cochlear use. These findings indicate that OCT2 blockade alone is insufficient to prevent cisplatin-induced damage in cochlear cells and highlight the need for a more comprehensive understanding of cisplatin transport dynamics in the inner ear. The interplay between uptake and efflux systems, such as OCT2, MATE1, and ATP-dependent transporters, may differ substantially from renal mechanisms. Future studies should aim to characterize these pathways in greater detail and evaluate alternative strategies for targeted modulation of cisplatin transport, with the goal of preventing ototoxicity without compromising antitumor efficacy.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

Ethical approval was not required for the studies on humans in accordance with the local legislation and institutional requirements because only commercially available established cell lines were used. The animal study was approved by Bioethics committee of the Faculty of Sciences of the University of Valparaiso. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

SW: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. CF-C: Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing, Software. HS: Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing, Supervision, Validation. AM: Data curation, Formal analysis, Investigation, Methodology, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing, Conceptualization, Funding acquisition, Resources.

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Conflict of interest

The author(s) declared that this work was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The author SW declared that they were an editorial board member of *Frontiers*, at the time of submission. This had no impact on the peer review process and the final decision.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fauot.2026.1729137/full#supplementary-material>

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