UNIVERSITAT DE BARCELONA

Model experimental de toxicitat còcleo-vestibular per administració intratimpànica de cisplatí en la rata

Àngela Callejo Castillo

NeuroToxicology 60 (2017) 1–9

Contents lists available at ScienceDirect

NeuroToxicology

Full Length Article

Dose-dependent cochlear and vestibular toxicity of trans-tympanic cisplatin in the rat

Angela Callejo[a,b,1], Amandine Durochat[c,1], Stéphanie Bressieux[c], Aurélie Saleur[c], Christian Chabbert[d], Ivan Domènech Juan[b,e], Jordi Llorens[a,e,f,*], Sophie Gaboyard-Niay[c,**]

[a] Departament de Ciències Fisiològiques, Universitat de Barcelona, 08907 L'Hospitalet de Llobregat, Catalonia, Spain
[b] Unitat Funcional d'Otorinolaringologia i Al·lèrgia, Institut Universtiari Dexeus, 08028 Barcelona, Catalonia, Spain
[c] Sensorion S.A., Montpellier, France
[d] Aix-Marseille Université, Marseille, France
[e] Institut de Neurociències, Universitat de Barcelona, Catalonia, Spain
[f] Institut d'Investigació Biomèdica de Bellvitge (IDIBELL), 08907 L'Hospitalet de Llobregat, Catalonia, Spain

ARTICLE INFO

Article history:
Received 27 June 2016
Received in revised form 10 February 2017
Accepted 10 February 2017
Available online 14 February 2017

Keywords:
Cisplatin
Ototoxicity
Hair cells
Vestibular and cochlear pathology
Trans-tympanic administration
Rat

ABSTRACT

In vivo studies are needed to study cisplatin ototoxicity and to evaluate candidate protective treatments. Rats and mice are the preferred species for toxicological and pharmacological pre-clinical research, but systemic administration of cisplatin causes high morbidity in these species. We hypothesized that trans-tympanic administration of cisplatin would provide a good model for studying its auditory and vestibular toxicity in the rat. Cisplatin was administered by the trans-tympanic route in one ear (50 μl, 0.5–2 mg/ml) of rats of both sexes and two different strains. Cochlear toxicity was corroborated by histological means. Vestibular toxicity was demonstrated by behavioral and histological analysis. Cisplatin concentrations were assessed in inner ear after trans-tympanic and i.v. administration. In all experiments, no lethality and only scant body weight loss were recorded. Cisplatin caused dose-dependent cochlear toxicity, as demonstrated by hair cell counts in the apical and middle turns of the cochlea, and vestibular toxicity, as demonstrated by behavioral analysis and hair cell counts in utricles. High concentrations of cisplatin were found in the inner ear after trans-tympanic administration. In comparison, i.v. administration resulted in lower inner ear concentrations. We conclude that trans-tympanic administration provides an easy, reproducible and safe model to study the cochlear and vestibular toxicity of cisplatin in the rat. This route of exposure may be useful to address particular questions on cisplatin induced ototoxicity and to test candidate protective treatments.

1. Introduction

Cisplatin is an effective chemotherapeutic agent widely used to treat a variety of malignant tumors. However, the use of cisplatin is limited by its three major side effects, nephrotoxicity, neurotoxicity and ototoxicity (Rybak, 2007; Ruggiero et al., 2013; Callejo et al., 2015). As other ototoxic compounds do, the damage caused by cisplatin progresses from the basal to the apical turn of the cochlea, thus causing high frequency hearing loss that extends to lower frequencies with increasing cumulative doses (Schacht et al., 2012; Langer et al., 2013). Although cisplatin ototoxicity has been extensively studied, many important questions remain unanswered, and no effective treatment is currently available to protect inner ear function in patients receiving cisplatin. One limiting factor in this research area is the shortcomings of the rodent models of cisplatin-induced ototoxicity. Due to its general toxic effects, most notably its renal toxicity, systemic cisplatin can result in a high rate of mortality (Chan et al., 2007; Minami et al., 2004; Wang et al., 2003). Moreover, the rapid alteration of the general state prevents proper assessment of the postural and motor alterations resulting from damage to the vestibule. In order to avoid the systemic side effects, co-administration of diuretics has been used to increase the susceptibility to cisplatin ototoxicity. These drugs increase the entry of ototoxic chemicals into the inner

* Corresponding author at: Departament de Ciències Fisiològiques, Universitat de Barcelona, c/Feixa Llarga s/n, 08907 L'Hospitalet de Llobregat, Spain.
** Corresponding author at: Sensorion S.A., 375 rue du Professeur Blayac, 34080 Montpellier, France.
E-mail addresses: jllorens@ub.edu (J. Llorens), gaboyard@yahoo.fr (S. Gaboyard-Niay).
[1] These two authors contributed equally to the study.

http://dx.doi.org/10.1016/j.neuro.2017.02.007
0161-813X/

ear after systemic administration (Li et al., 2011; Ding et al., 2012). Other authors have designed local administration models to study cisplatin-induced cochleotoxicity. However, most of these models include a surgical procedure that implies some morbidity and experimental difficulty (Janning et al., 1998; Korver et al., 2002; Whitworth et al., 2004; He et al., 2009; Xia et al., 2012). Local administration of drugs or gene vectors can be achieved by the transtympanic route (Sera et al., 1987; Llorens et al., 1993; Llorens and Rodríguez-Farré, 1997; Mukherjea et al., 2011; Rúa et al., 2013), which is much less invasive and is in fact increasingly used in clinical practice (Salt and Plontke, 2009; Miller and Agrawal, 2014). To our knowledge, the study by He et al. (2009) in the mouse is the only one using trans-tympanic administration of cisplatin. This study does not include a dose-response assessment. Also, the size of the mouse makes the trans-tympanic administration more difficult than its application to larger animals such as the rat.

In addition to the auditory damage, most ototoxic compounds cause also vestibular dysfunction. The susceptibility of the vestibular system to cisplatin toxicity has not been clearly established. Vestibular hair cells (HC) have been found to be sensitive to cisplatin ototoxicity at concentrations close to those causing degeneration of cochlear outer HCs (OHCs) *in vitro* (Zhang et al., 2003; Cunningham and Brandon, 2006; Schmitt et al., 2009). However, clinical data indicate a low incidence of vestibular dysfunction following cisplatin administration (Strauss et al., 1983; Myers et al., 1993; Madasu et al., 1997). Nevertheless, vestibular effects have been reported in several studies (Schaefer et al., 1981; Wright and Schaefer, 1982; Kobayashi et al., 1987; Kitsigianis et al., 1988; Prim et al., 2001). Few animal studies have assessed the vestibular toxicity caused by systemic cisplatin exposure, and both null and small effects have been recorded (Schweitzer et al., 1986; Sergi et al., 2003; Caston and Doinel, 1987; Laurell and Bagger-Sjöbäck, 1991; Nakayama et al., 1996; Lo et al., 2015). As an exception, Tian et al. (2013), reported significant auditory and vestibular toxicity in rats following intraperitoneal exposure to cisplatin in a high volume of saline. Also, behavioral evidences of vestibular dysfunction have been reported in animals following local cisplatin exposure for cochlear studies (Janning et al., 1998; Korver et al., 2002), but none of these specifically addressed the vestibular effect.

The experiments presented here were aimed at evaluating the consequences of trans-tympanic administration of cisplatin in the rat. Specific aims were 1) to evaluate whether this is an easy, short, and reproducible technique to study cisplatin-induced dose-dependent ototoxicity; 2) to test its consequences in terms of morbidity; 3) to assess its vestibular toxicity through behavioral and histological approaches.

2. Materials and methods

2.1. Chemicals

Cisplatin was obtained from Sigma-Aldrich (Madrid, Spain) or EDQM (Strasbourg, France). For immunolabeling, we used the following antibodies: rabbit anti-calbindin (CB-38a, Swant, used at 1/1000 final dilution), rabbit anti-myosin VIIa (MYO7A) (25-6790, Proteus Biosciences, 1/700 or 1/1000), and mouse monoclonal anti-neurofilament 200 kD (clone N52, Sigma, 1/1000). Secondary antibodies were Alexa-Fluor 488-conjugated donkey anti-rabbit, Alexa-Fluor 647-conjugated donkey anti-mouse and Alexa-Fluor 594-conjugated donkey anti-rabbit (Invitrogen, 1/500 or 1/700). We also used Alexa-Fluor 488-conjugated phalloidin and Alexa-Fluor 555-conjugated phalloidin (1/1000 or 1/100, respectively) to label actin.

2.2. Animals

Male (225–249 g) or female (150–200 g) Long-Evans rats, and male and female Wistar (225–250 g) rats, were all obtained from Janvier (Le Genest St Isle, France) at least one week before experimentation. They were housed two or three per cage in standard Macrolon cages with wood shavings as bedding, and were maintained on a 12:12L:D cycle at 22 + 2 °C. Water and standard diet pellets were available *ad libitum*.

Male Long-Evans experiments were performed at the premises of the Bellvitge Campus of the Universitat de Barcelona. Other experiments were performed at the Institut des Neurosciences de Montpellier. In either site, the animals were handled according to the EU Directive 86/609/EEC as implemented through the locally applicable laws. The experiments were performed according to the following protocols approved by the corresponding Ethic Committee on Animal Experimentation: number 335/09 (Principal Investigator J. Llorens) by the committee of the Universitat de Barcelona, and number LR-12140 (Principal Investigator S. Gaboyard-Niay) by the committee of the Languedoc-Roussillon.

2.3. Cisplatin exposure

Rats were anesthetized with isoflurane. Applying traction to the auricle, the posterior portion of the tympanic membrane was penetrated with a sterile insulin syringe (0.33 × 12.7 mm or 0.5 × 16 mm). Cisplatin, dissolved in 50 μl of saline, was slowly injected into the middle ear cavity. We used cisplatin solutions at final concentrations of 0.5–2.0 mg/ml, which resulted in total amounts from 25 to 100 μg of cisplatin per injection. These concentrations were selected around the single dose concentration (1 mg/ml) used in the study by He et al. (2009) in the mouse. In some animals the second ear served as non-injected control, while in others it was injected with saline. Animals receiving no cisplatin and only saline in one side were evaluated to exclude adverse effects of the injection procedure, but these were not included in the analyses. Comparisons were made between cisplatin ears and contra-lateral control ears. After each injection, the animal was kept in lateral position for 10–15 min under isoflurane, and observed until recovered from the anesthesia. Male Long-Evans rats were administered 0.5, 0.75, 1.0, 1.5, or 2.0 mg/ml (n = 4–6/group), evaluated for vestibular dysfunction, and processed for cochlear histology. Female Long-Evans and male Wistar rats were administered 0.5, 1.0, or 2.0 mg/ml (n = 7–8/group), evaluated for vestibular dysfunction, and processed for utricle histology. Female Wistar rats were exposed to 2.0 mg/ml (n = 16), evaluated for vestibular dysfunction, and processed for utricle histology.

To obtain a preliminary clue of the role of pharmacokinetic factors in the susceptibility of rats to cisplatin ototoxicity by trans-tympanic exposure, twelve additional female Long-Evans rats were used to compare cisplatin pharmacokinetics after systemic and trans-tympanic administration. Eight rats received a single intravenous (i.v.) dose of cisplatin (10 mg/kg at 10 ml/kg in 5 min) via the tail vein. This dose was selected to match a widely used human dose (60 mg/m^2) following standard methods for animal to human dose conversion (Nair and Jacob, 2016). Four rats received a trans-tympanic injection of 50 μl (2.0 mg/ml cisplatin) as described above.

2.4. Animal evaluation

The body weight and behavior of the animals were assessed on days 0 (pre-test), 1, 2, 3, 4 and 7 after trans-tympanic administration. Behavioral assessment of vestibular dysfunction in male Long-Evans was performed using the test battery described by Vignaux et al. (2012) for unilateral lesions. Other experiments used

an adapted version of a test battery initially developed for bilateral lesions (Boadas-Vaello et al., 2005; Llorens and Rodríguez-Farré, 1997; Llorens et al., 1993). In both cases, altered vestibular behaviors were scored on a scale from 0 to 4, respectively ranging from normal behavior (rating 0) to maximal identified vestibular impairment (rating 4). Five different tests were sequentially scored and summed to rate the vestibular disorder. The Vignaux et al. (2012) test battery included: 1- head tilt (unilateral vestibular deficit induces postural head deviation); 2- body twisting (starts with head rotation around the longitudinal axis of the body which triggers body rotation in a single deviation); 3- walking deviation towards one side; 4- tail-hanging reflex (normally induces a forelimb extension to reach the ground, unilateral disorder results in axial rotation of the body); 5- tail rotation (rotation of the tail observed by holding the rat's body above a table surface. The second test battery included: 1- head tilt (as above); 2- circling (stereotyped movement ranging from none to compulsive circles around the hips of the animal); 3- head bobbing (abnormal intermittent backward extension of the neck); 4- tail-hanging reflex (as above); 5- air-righting reflex (necessary for rats to land on all four feet when falling from a supine position; vestibular dysfunction impairs normal body repositioning with a maximal disorder leading the animal to land on its back when dropped from a height of 40 cm onto a foam cushion). Vestibular dysfunction scores are expressed as median values.

2.5. Inner ear preparation and immunohistochemistry

On day 3 or 7 after transtympanic injection, rats were anesthetized with isoflurane or pentobarbital (100 mg/kg, i.p.) and the tympanic membranes were inspected again with a microscope to exclude tympanic perforation and acute otitis media. For histology investigation, after decapitation, temporal bones were quickly collected and placed in 4% freshly depolymerized paraformaldehyde in phosphate buffered saline (PBS) for immediate dissection.

For cochlear histology, a small hole was made in the apex of the cochlea and fixative was flushed into the cochlea at the beginning of the dissection. The cochlear dissection reliably provided six consecutive 1/4 turn sections from the apex to the middle of the second turn. After dissection, the cochlear tissues were fixed for 1 h at room temperature in the same fixative and then stored at −20 °C in a cryoprotective solution (34.5% glycerol, 30% ethylene glycol, 20% PBS, 15.5% distilled water) until processed for whole-mount immunohistochemistry. Tissues were first permeabilized and blocked for 90 min in 4% Triton-X-100 and 20% donkey serum in PBS. Primary antibodies were incubated in 0.1% Triton-X-100 and 1% donkey serum in PBS for 48 h at 4 °C. Secondary antibodies and phallotoxins were incubated together in 0.1% Triton-X-100 in PBS overnight at 4 °C. Specimens were thoroughly rinsed with PBS between incubations. After immunolabeling, sections were mounted in Mowiol (Sigma) medium.

Utricles were obtained, fixed as above for 1 h and then processed for whole-mount immunohistochemistry. Sensory epithelia were first permeabilized with 4% Triton X-100 in PBS, under rotation for 1 h at RT. Non-specific binding was then prevented by a pre-incubation step in a blocking solution of 0.5% fish gelatin and 0.5% Triton X-100 in PBS, under rotation for 1hr at RT. Samples were then incubated with primary antibodies anti-MYO7A diluted in the blocking solution rotating for 48 h at 4 °C. Specific MYO7A labeling was revealed with Alexa 488-conjugated secondary antibodies, incubated in blocking solution, rotating overnight at 4 °C. This incubation included also Alexa 594-conjugated phalloidin for actin staining. Extensive PBS rinses were performed between each labeling step. Whole mount utricles were mounted on slides in Mowiol (Calbiochem, Darmstadt, Germany). The primary antibodies we used are highly specific and well characterized antibodies (Flores-Otero et al., 2007; Golub et al., 2012; Rúa et al., 2013; Seoane et al., 2003; Soler-Martín et al., 2014). Omission of the primary antibody was used as control to confirm the specificity of the immunolabel.

2.6. Imaging and morphometry

Cochlear segments were observed in a Zeiss Jenalumar fluorescence microscope and a Leica TCS-SL confocal microscope. Images spanning 10 inner HC (ICH) were obtained from each cochlear segment and the number of OHC remaining in that section was determined by simple visual inspection. Counts were obtained from four to six specimens per dose level and cochlear level, and from 20 untreated control cochleas from the same animals.

Utricle samples were observed with a Zeiss Apotome Fluorescence Microscope. Two stacks (Obj 40×), one from striolar zone and one from extrastriola were imaged per utricle. MYO7A and phalloidin labels were recorded separately in two different color channels, and used to count hair cells or hair bundles, respectively, using ImageJ software. A minimum of three epithelia per gender and strain were quantified. Final image processing was done with Adobe Photoshop software (San Jose, CA).

2.7. Pharmacokinetics

Animals were killed under terminal anesthesia (pentobarbital, 100 mg/kg). Terminal blood was sampled by intracardiac puncture and the right temporal bone was excised at 30 or 90 min after end of i.v. administration. Temporal bones at 90 min were obtained from rats given trans-tympanic cisplatin. Blood samples were collected in heparinized tubes (Teklab Ltd, Durham, UK) and centrifuged to obtain plasma. Temporal bones and plasma samples were stored at −20 °C until transportation (dry ice) and analysis by the Analytical Department of Synovo GmbH (Tübingen, Germany). To obtain inner ear samples for analysis, temporal bones were thawed and the inner ear exposed by dissection. Upon exposure of the tip of the cochlea, ca. 3 μl of perilymph or fluid could be recovered. The bones of the inner ear and cochlea components were removed with fine forceps and added to the perilymph in an Eppendorf tube. This protocol prevented contamination of the samples by blood or cerebrospinal fluid. 8 μl of water was added to the combined collection. After mixing 5 min in an ultrasound bath, 30 μl of acetonitrile containing internal standard was added to each sample tube. After mixing and incubating 30 min, the samples were centrifuged and the supernatant was transferred to HPLC glass vials. The absence of fat and the incubation in solvent is intended to provide sufficient solubility of the test articles. The concentrations of cisplatin in the plasma and inner ear were determined by high performance liquid chromatography/tandem mass spectrometry (HPLC/MS/MS). The LC system comprised an Agilent 1260 binary pump, an auto-sampler and a column oven. The chromatography column was ReproSil Pur C18-AQ. The detector was an AB SCIEX API 4000 (triple-quadrupole) instrument with an ESI interface (Mass spectrometric analysis). The data acquisition and control was via Analyst version 1.6.2. from Applied Biosystems Inc.

2.8. Statistical analysis

IBM SPSS Statistics 20.0 or SigmaPlot (Systat Software, Chicago, IL) were used for statistical analysis. Body weight data were analyzed by repeated measures analysis of variance (MANOVA). Body weight data were also analyzed by ANOVA with day as factor. Behavioral scores of vestibular dysfunction were analyzed by Friedman's non-parametric repeated measures analysis of variance

(RM-ANOVA). Day by day dose group comparisons were assessed by Kruskall-Wallis analysis of variance, followed by Mann-Whitney *U* test for selected pair-wise comparisons. For vestibular morphometry, two way analysis of variance (ANOVA) was used for an overall comparison of the effect of the cisplatin treatment across the different strain, sex and time point groups; this was followed by two-sided Student's *t*-tests to compare treated versus control sides within each group. Cochlear data were analyzed by Kruskall-Wallis non-parametric analysis of variance.

3. Results

3.1. Trans-tympanic cisplatin cause only minor systemic toxicity

Rats exposed to cisplatin by unilateral trans-tympanic administration showed no mortality and little evidence of systemic toxicity. At the highest dose (2 mg/ml, 100 μg in 50 μl), slight piloerection and diarrhea was noticed in some animals up to day 3 after dosing, but this resolved afterwards. Fig. 1 shows body weight data from male Long-Evans rats. MANOVA analysis revealed a significant day effect, $F(4,14) = 16.6$, $p < 0.001$, but no significant treatment effect, $F(4,17) = 1.79$, $p = 0.178$, nor treatment by day interaction effect, $F(16,43) = 1.6$, $p = 0.108$, indicating an absence of major effects of the treatment on body weight. The maximum difference between initial and post-treatment mean body weights was recorded at day 3 after treatment, when the group given 1 mg/ml cisplatin showed a 96.7% of the initial body weight. The variations in body weights across days were not significant (all F's < 1.3, p's > 0.3), and mean values at day 7 they were higher than initial mean weights in all treatment groups. An absence of major effects on body weights was similarly obtained in all other animal sex and strains (Supplementary Fig. 1). In all cases, group mean body weights remained above 94% of the pre-treatment values at all times after dosing and showed recovery by day 7 after dosing.

3.2. Trans-tympanic cisplatin cause dose-dependent loss of vestibular function

Trans-tympanic injection of cisplatin caused a dose-dependent loss of vestibular function, as assessed by a test battery that evaluates postural, spontaneous and reflex behaviors. As shown in Fig. 2A, male Long-Evans rats receiving 50 μl of 1.0, 1.5 or 2.0 mg/ml of cisplatin in one ear showed evidence of unilateral vestibular dysfunction progressing from day 1–3 after dosing with little evidence for recovery up to day 7. Lower doses of cisplatin caused a small transient (0.75 mg/ml) or null (0.50 mg/ml) effect on the vestibular dysfunction scores. Friedman's repeated measures analysis of variance revealed statistically significant effects of the 0.75 ($chi^2_{(5)} = 11.08$, $P = 0.05$), 1.0 ($chi^2_{(5)} = 16,89$, $P = 0.005$), and 1.5 mg/ml ($chi^2_{(5)} = 15.62$, $P = 0.008$) doses. Comparisons across dose groups indicated statistically significant differences at days 1 ($P = 0.031$), 2 ($P = 0.012$), and 3 ($P = 0.025$), but not at days 4 and 7 ($P = 0.055$ in both cases). A dose-dependent loss of vestibular function was also recorded in female Long-Evans (Fig. 2B). All dose effects were significant ($P = 0.037$, $P = 0.001$, and $P < 0.001$, for the 0.5, 1.0, and 2.0 mg/kg doses, respectively), and differences between dose groups were found at all days after treatment ($P \leq 0.001$). Dose-dependent effects were also recorded in male Wistar rats (data not shown). Wistar females were evaluated at the 2 mg/ml dose only, and altered vestibular function was also recorded. Fig. 2C shows the results obtained for this dose of cisplatin up to day 3 in the four strain and sex groups. In Wistar male and female rats, and Long-Evans females, a significant change in behavioral scores across time was recorded ($P < 0.001$) while

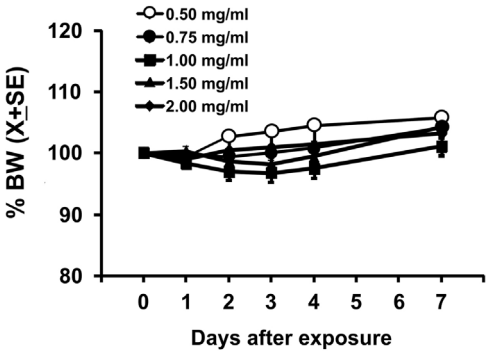

Fig. 1. Effects of trans-tympanic cisplatin (50 μl of 0.5, 0.75, 1.0, 1.5, or 2.0 mg/ml, unilateral) on body weight (X ± SE) of male Long-Evans rats (n = 4–6/group). Error bars are not visible when they are smaller than point size.

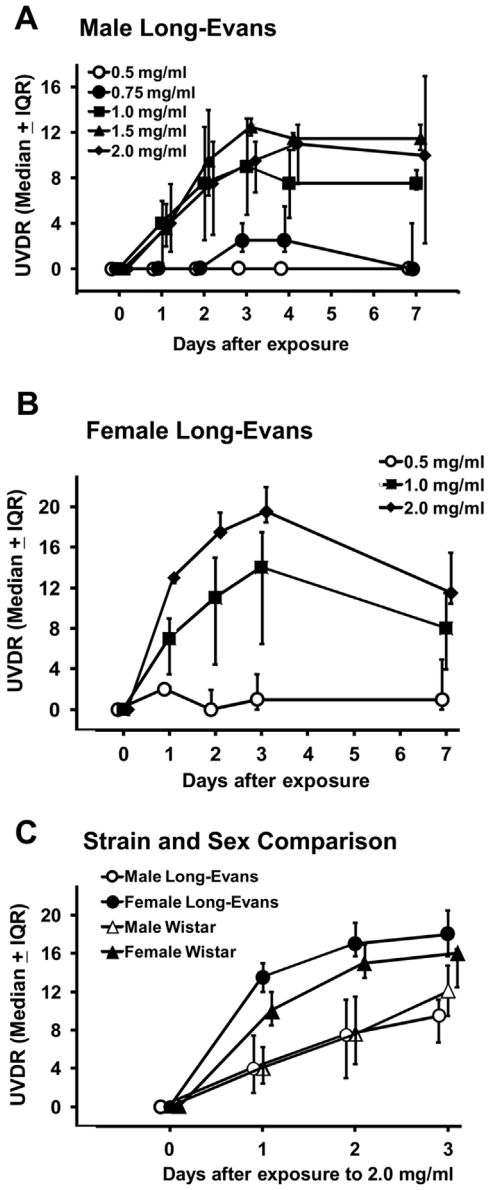

Fig. 2. Effects of unilateral trans-tympanic cisplatin on scores of unilateral vestibular dysfunction in rats (see the Methods section for further details). Data are median ± interquartile range (IQR). A: Effects of 0.5, 0.75, 1.0, 1.5 or 2.0 mg/ml in male Long-Evans rats (n = 4–6/group). B: Effects of 0,5, 1.0 or 2.0 mg/ml in female Long-Evans rats (n = 8/group). C: Effects of 2.0 mg/ml in male and female Long-Evans and Wistar rats (n = 4-16/group).

Long-Evans males did not show significant differences ($P = 0.097$). Day by day comparisons resulted in significant group differences (all $P < 0.001$), and these were associated with a higher effect in

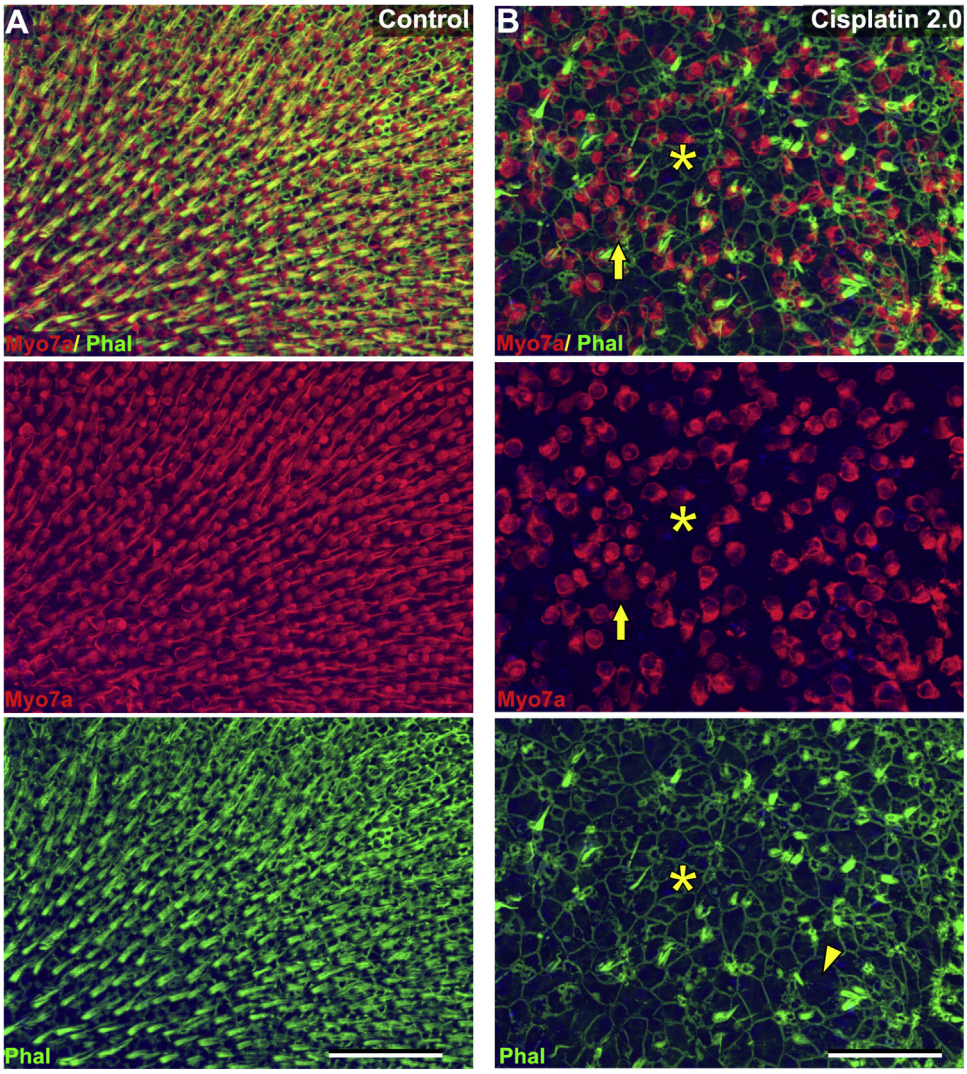

Fig. 3. Loss of hair cells in the utricle after trans-tympanic cisplatin administration (50 μl, 2 mg/ml). A and B: Representative images of a control (A) and a treated (B) utricle at 7 days after exposure in male Wistar rats. Epithelia were immunolabeled with anti-MYO7A antibody (red) to stain hair cells, and labeled with phalloidin (green) to stain hair bundles. For illustrative purposes, the upper panels show the two stains superimposed with co-localization shown in yellow. In B, note i) the increased surfaces of the supporting cells delineated by actin rings (arrowhead); ii) the low density of HCs, including large areas containing no HCs (asterisks); and iii) the abnormal shapes of many of the HCs that remain in the utricle. Scale bars = 50 μm. C: Number of MYO7A-positive cells in utricles after cisplatin administration, expressed as percent of control mean values (n = 3–8/group, side, and utricle zone). * $p < 0.05$, different from control side, Student's *t*-test. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

female compared to male rats ($P < 0.001$ at all three days after exposure).

3.3. Trans-tympanic cisplatin cause loss of vestibular HCs

The toxicity of trans-tympanic cisplatin on the vestibular epithelia was assessed in rats exposed to the 2 mg/ml dose. Fig. 3 compares a control utricle (Fig. 3A) and a utricle from an ear of male Wistar rats treated with cisplatin (Fig. 3B), the latter showing overt loss of HCs. This loss was clearly observed in both MYO7A-positive HC and phalloidin-labeled hair bundles. Fig. 3C displays the mean percent values respect to control HC counts, obtained in female Wistar rats at 3 days after injection and in female Long-Evans and male Wistars at 7 days. The data showed extensive (50–80%) loss of vestibular HCs. Similar results were obtained with counts of phalloidin-stained hair bundles (data not shown). Statistically significant differences were recorded in all comparisons between HC counts from cisplatin injected and control ears. The recorded statistics were: 1) $T_6 = 8.3$, $P < 0.001$, in male Wistar whole utricle at 7 days; 2) $T_7 = 7.3$, $P < 0.001$, in female Long Evans whole utricle at 7 days; $T_{12} = 4.4$, $P = 0.001$, in female Wistar whole utricle at 3 days; $T_5 = 5.5$, $P = 0.03$, in female Wistar striola region at 3 days; and $T_5 = 5.6$, $P = 0.02$, in female Wistar extrastriola region at 3 days. A two-way ANOVA analysis with experimental group and treatment as factors resulted in a treatment effect of $F_{(1,35)} = 132$, $P < 0.001$. Therefore, the loss of vestibular function recorded after trans-tympanic administration of cisplatin was associated with a loss of vestibular HCs.

3.4. Trans-tympanic cisplatin cause dose-dependent loss of cochlear OHCs

Morphological assessment of the cochlear toxicity was performed at 7 days after administration in male Long-Evans rats. Cisplatin was found to cause overt cochlear damage (Figs. 4 and 5). Control specimens showed a complete set of IHCs and OHCs (Fig. 4A). Ears administered with cisplatin showed no loss of IHCs in the cochlear region selected for study, the apical 1 $^1/_2$ turns. These specimens showed a reduction of OHCs number to an extent that depended both on the dose and the cochlear distance from the apex. The OHC loss ranged from moderate (Fig. 4B) to complete (Fig. 4C). To assess the cisplatin toxicity, OHC counts from the apical 1 $^1/_2$ turns of the cochlea were obtained (Fig. 5). Similar lesions were observed in the animals given 1, 1.5 or 2 mg/ml of cisplatin, in which only the most apical half turn of the cochlea contained a noticeable mean number of OHC, while the lower half of the apical turn and the upper half of the middle turn contained almost no OHCs remaining. Animals given 0.75 mg/ml cisplatin showed a progressive loss of OHC between the second quarter of the apical turn, where a median of 25 OHC were counted in the 10 IHC segment, and the second quarter of the middle turn, where this median number was of only 2 cells. The rats receiving the lower dose of cisplatin (0.5 mg/ml) showed a smaller effect on OHC, but a noticeable loss was nevertheless observed at the second quarter of the middle turn, where only a median number of 22.5 cells were present compared to the 30 OHC found in the intact control cochlea. Kruskall-Wallis analysis of variance resulted in significant group differences in OHC numbers in all cochlear segments. The recorded statistics were as follows, in descending order from the apical end segment: 1) $chi^2_{(5)} = 14.69$, $P = 0.012$; 2) $chi^2_{(5)} = 26.50$, $P < 0.001$; 3) $chi^2_{(5)} = 27.53$, $P < 0.001$; 4) $chi^2_{(5)} = 33.04$, $P < 0.001$; 5) $chi^2_{(5)} = 30.61$, $P < 0.001$; 6) $chi^2_{(5)} = 32.28$, $P < 0.001$.

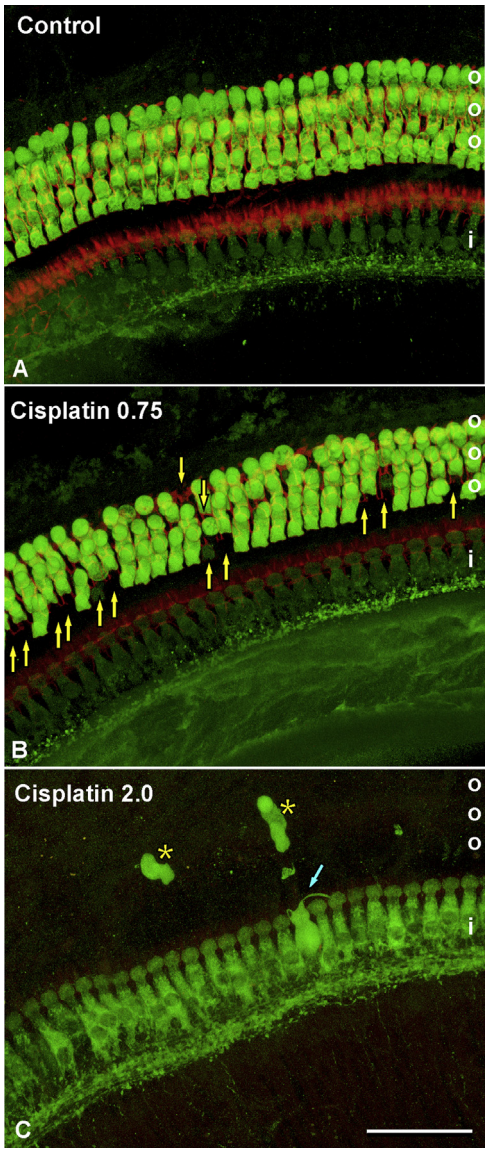

Fig. 4. Loss of cochlear OHCs after trans-tympanic cisplatin administration. Representative images from the 3rd quadrant of the apical turn at 7 days after administration. Hair cells were immunolabeled with a mixture of anti-calbindin and anti-MYO7A antibodies (green); hair bundles and actin rings were labeled with phalloidin (red). (A) Control. Note the uniform distribution of hair cells in one row of IHCs (i) and three rows or OHCs (o). (B) Noticeable loss of OHCs (arrows) after 0.75 mg/ml of cisplatin. (C) Only two degenerating OHCs (asterisks) remain in this cochlear segment after 2 mg/ml of cisplatin. One of the IHCs is abnormally swollen (arrow). Scale bar = 25 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.5. High concentrations of cisplatin are attained in the inner ear after trans-tympanic exposure

Cisplatin concentrations in plasma and inner ear after intravenous or trans-tympanic exposure are shown in Table 1. After systemic exposure, plasma but not ear cisplatin concentrations decreased between 30 and 90 min. The ear concentrations at 90 min after trans-tympanic administration were one order of magnitude larger than those recorded 30 or 90 min after intravenous exposure.

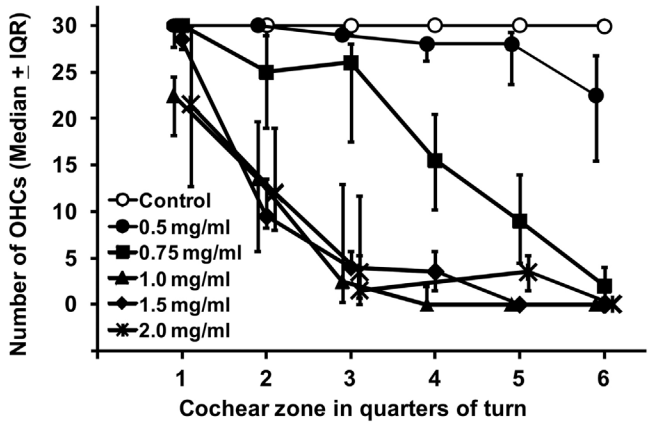

Fig. 5. Loss of cochlear OHCs after trans-tympanic cisplatin administration. Median ± interquartile range (IQR) OHC counts are shown for a 10 IHC-wide segment from the 6 apical-most quadrants of the cochlea, from the apical end to half of the second turn. Control data are from uninjected control sides (n = 20). Data points for cisplatin are from 4 to 6 ears per group.

4. Discussion

In this study, the ototoxicity of cisplatin has been evaluated in a trans-tympanic exposure model in the rat. Cisplatin ototoxicity is well recognized (Rybak, 2007) and, if available, prophylactic treatments not interfering with its antineoplasic effects would be useful to prevent this undesirable side effect. To develop such protective drugs, *in silico*, molecular and *in vitro* studies must be valued by animal studies to validate and assess the protective efficacy *in vivo*. These studies become problematical because of the systemic toxicity of cisplatin. Thanks to the ear's anatomy, local application may be used to circumvent these effects. Although several studies are available describing the ototoxicity of cisplatin after local application (Janning et al., 1998; Korver et al., 2002; Whitworth et al., 2004; He et al., 2009; Xia et al., 2012), none of them have examined the cochlear and vestibular effects of trans-tympanic administration in the rat. After trans-tympanic administration, drugs can directly access to the perilymph across the round window membrane (Plontke et al., 2008). However, literature data indicates that substances can enter into the inner ear also through the oval window (Zou and Pyykkö, 2013). In an animal study with guinea pigs, gentamicin was applied on the round window or around the staples footplate on the oval window, and the latter exhibited markedly higher levels of hearing loss and a greater reduction of cochlear and vestibular cells (King et al., 2013). We reasoned that trans-tympanic administration would circumvent most of the difficulties caused by the systemic toxicity, and yet allow evaluating cisplatin ototoxicity in the intact inner ear. By flooding the middle ear, the drug would reach the annular ligament of the oval window and enter the inner ear, with more efficient results than those models that administered the drug only on the round window following surgical opening of the bulla. While this route of administration eludes the role of the ear vascularity and blood-inner ear barrier in the toxicity, it keeps all other variables, such as the presence of the endolymphatic compartment, the afferent and efferent innervations, and the continued physiological stimulation of the sensory epithelia. The present demonstration of ototoxic effects of cisplatin following middle ear exposure demonstrates its permeation into the inner ear, in good accordance with its cationic properties and the evidence that cationic substances can easily pass through the round window membrane (Goycoolea and Lundman, 1997).

The data obtained indicated that unilateral trans-tympanic cisplatin causes scarce systemic toxic effects, besides the ear toxicity. All groups showed effects on body weight of less than 6% loss, with rapid recovery within a week. This limited toxicity compares favorably with the high rates of mortality and much deeper loss of weight that is commonly found after systemic exposure (He et al., 2009; Madias and Harrington, 1978; Ding et al., 2012). Therefore, trans-tympanic exposure provides a model to study cisplatin ototoxicity with low morbidity in the rat. This adds value to a technique that after a short training becomes easy, short, and reproducible. Although the puncture of the tympanic membrane can eventually cause permanent tympanic perforation and middle ear infection, wound healing and absence of signs of infection were recorded in this study, in agreement with literature data (Mukherjea et al., 2011). These favorable data in the rat contrast with the high rates of tympanic membrane perforation and middle ear damage reported by He et al. (2009) in the mouse, a difference explained by the difficulties in the procedure caused by the small size of the latter species.

Trans-tympanic cisplatin caused dose-dependent vestibular and cochlear toxicity. In the cochlea, we observed a complete or almost complete loss of OHCs from the middle of apical turn downwards and a major loss in the most apical half turn after 1.0–2.0 mg/ml of cisplatin. The loss of cochlear OHCs started in more basal regions at lower doses, 0.75 and 0.5 mg/ml, resulting in a better preservation of the apical cochlea. We did not examine high frequency hearing or quantify basal parts of the cochlea, but the observations made on these parts when available indicated that the well known basal to apical gradient of toxicity was present throughout the entire cochlea. Therefore, it is likely that concentrations below 0.5 mg/ml, not evaluated in this series of experiments, cause significant damage in the basal cochlea in this trans-tympanic rat model. In the literature, significant cochlear toxicity has been reported after exposure to: (1) 1 mg/ml (trans-tympanic in the mouse; He et al., 2009); (2) 0.25 mg/ml (round window application in the chinchilla; Janning et al., 1998); (3) 0.66 mg/ml (round window application in the chinchilla; Whitworth et al., 2004); and (4) 0.25 and 0.50 mg/ml (round window in the guinea pig).

We also recorded a dose-dependent loss of vestibular function, with none to mild effects at 0.5 and 0.75 mg/ml and marked vestibular dysfunction at 1.0–2.0 mg/ml. This loss of function was demonstrated by a well established set of behavioral tests previously demonstrated to be specific and reliable to assess vestibular dysfunction (Llorens et al., 1993; Boadas-Vaello et al., 2005; Brugeaud et al., 2007; Vignaux et al., 2012; Saldaña-Ruíz et al., 2013; Dyhrfjeld-Johnsen et al., 2013; Gaboyard-Niay et al.,

Table 1
Cisplatin concentrations in plasma and inner ear tissue after systemic (intravenous, i.v.) or trans-tympanic administration in female Long Evans rats. Mean concentrations (±SE; n = 4 animals per time point) are expressed in μg/ml. Comparative Human Equivalent Dose (HED) is given as referential.

Dose		Stock	Route	Plasma		Inner ear	
Rat	HED			30′	90′	30′	90′
10 mg/kg	60 mg m^2	1 mg/ml	i.v.	3.7 ± 0.1	1.3 ± 0.2	2.5 ± 0.1	1.4 ± 0.1
0.1 mg/ear	n.a.	2 mg/ml	trans-tympanic	n.a.	n.a.	n.a.	19.4 ± 7.6

2016).The vestibular toxicity of cisplatin *in vivo* was also demonstrated by histological analysis. We recorded loss of utricular HCs after intra-tympanic exposure to 2.0 mg/kg of cisplatin, ranging between 50 and 80% in the different estimates according to strain, sex, and time. We thus confirm the *in vitro* (Zhang et al., 2003; Cunningham and Brandon, 2006; Schmitt et al., 2009) and animal data (Janning et al., 1998; Korver et al., 2002; Sergi et al., 2003; Tian et al., 2013; Lo et al., 2015) indicating that cisplatin causes vestibular toxicity in addition to cochlear toxicity.

The vestibular toxicity of cisplatin was recorded in two strains of rats and in both sexes. Although the experiments were not designed for direct strain and sex comparisons, the data obtained suggest that both strains are similarly susceptible while female rats may be more sensitive than male rats to the vestibular toxicity of cisplatin. Although females had lower body weights, the local nature of the administration makes it unlikely that the difference in dose on a body weight basis caused a difference in toxicity. Additional experiments will be necessary to confirm a sex effect in this model, and eventually to investigate the underlying mechanisms. In relation to this point, it is worth noting that a higher susceptibility of females compared to males has been recently reported for the auditory toxicity of cisplatin following systemic administration in rats (Kirkim et al., 2014).

The robustness of the vestibular effect recorded in this study likely relies on the high concentrations of cisplatin attained in the inner ear after trans-tympanic administration of cisplatin. By comparison, remarkably smaller concentrations were attained following systemic (i.v.) exposure. Taken together, the present and previous data indicate that cisplatin is toxic to the vestibular HCs when effective concentrations are attained in the inner ear, but that these concentrations may be not attained after a single i.v. dose. Therefore, the lack of vestibular effects recorded in a number of animal studies most likely results from the use of dosing regimens attaining only sub-threshold concentrations. The present behavioral data showed vestibular toxicity starting at 0.75 mg/ml, in parallel with a loss of OHCs being already extensive in the middle turn of the cochlea. For comparison, significant cochlear toxicity has been reported in chinchillas after exposure to 0.25 mg/ml (Janning et al., 1998). Additional studies are required to provide a more extensive characterization of the comparative auditory and vestibular toxicity of cisplatin in this exposure model.

Cisplatin was approved for clinical use in 1978, and is nowadays the main option in the treatment of a wide variety of cancers. Since then, hearing loss as a major adverse effect has been well described in adult and children cancer patients (Grewal et al., 2010; Bokemeyer et al., 1998). In contrast, vestibular toxicity was reported early (Schaefer et al., 1981; Wright and Schaefer, 1982) but has remained rarely reported as a direct adverse effect of cisplatin (Kobayashi et al., 1987; Kitsigianis et al., 1988; Prim et al., 2001; Gurney et al., 2007; Tofthagen et al., 2012), while several studies have indicated absence of vestibular effects in cisplatin patients (Strauss et al., 1983; Myers et al., 1993; Madasu et al., 1997). However, recent clinical studies have highlighted vestibular symptoms in cancer survivors such as enhanced risk of falls after chemotherapy linked to loss of balance in adults (Tofthagen et al., 2012) or persistent dizziness in children (Gurney et al., 2007). The trans-tympanic rat model presented here may allow comparing the vestibular and auditory toxicities of cisplain and the effectiveness in both systems of prophylactic treatments aimed at reducing cisplatin ototoxicity.

In conclusion, we present here initial data on the auditory and vestibular effects of trans-tympanic cisplatin administration in the rat. Although the data presented are limited in several aspects, including the lack of auditory functional assessment, they allow concluding that this exposure model causes: 1) low morbidity; 2) dose-dependent cochlear toxicity; and 3) dose-dependent vestibular toxicity. Although this route of exposure is not the one humans are exposed to, it may be useful as an experimental model to answer particular questions on cisplatin ototoxicity and to test candidate protective treatments. In comparison to *in vitro* studies, this model allows the study of the intact systems. In comparison to systemic exposure models, it circumvents the problems caused by the systemic toxicity of the drug.

Conflicts of interest

A.D., S.B., A.S., and S.G.-N. work for Sensorion, a biopharmaceutical company committed to finding treatments for inner ear disease. S.B., A.S., S.G.-N and C.C. have shares in this company. C.C. is also a consultant for Sensorion. Other authors declare that they have no conflict of interest.

Acknowledgements

The cochlear confocal microscopy studies were performed at the Scientific and Technological Centers of the University of Barcelona (CCiT-UB). We thank Benjamín Torrejon-Escribano for advice on confocal imaging. This study was supported by the Ministry of Economy and Competitiveness (Spain)/European Regional Development Fund (European Union) [BFU2012-31164, and BFU2015-66109-R]; and by the Agència de Gestió d'Ajuts Universitaris i de Recerca (Catalonia) [2014 SGR 943].

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.neuro.2017.02.007.

References

Boadas-Vaello, P., Riera, J., Llorens, J., 2005. Behavioral and pathological effects in the rat define two groups of neurotoxic nitriles. Toxicol. Sci. 88, 456–466.
Bokemeyer, C., Berger, C.C., Hartmann, J.T., Kollmannsberger, C., Schmoll, H.J., Kuczyk, M.A., Kanz, L., 1998. Analysis of risk factors for cisplatin-induced ototoxicity in patients with testicular cancer. Br. J. Cancer 77, 1355–1362.
Brugeaud, A., Travo, C., Demêmes, D., Lenoir, M., Llorens, J., Puel, J.-L., Chabbert, C., 2007. Control of hair cell excitability by vestibular primary sensory neurons. J. Neurosci. 27, 3503–3511.
Callejo, A., Sedó-Cabezón, L., Domènech Juan, I., Llorens, J., 2015. Cisplatin-induced ototoxicity: effects, mechanisms and protection strategies. Toxics 3, 268–293.
Caston, J., Doinel, L., 1987. Comparative vestibular toxicity of dibekacin, habekacin and cisplatin. Acta Otolaryngol. 104, 315–321.
Chan, D.K., Lieberman, D.M., Musatov, S., Goldfein, J.A., Selesnick, S.H., Kaplitt, M.G., 2007. Protection against cisplatin-induced ototoxicity by adeno-associated virus-mediated delivery of the X-linked inhibitor of apoptosis protein is not dependent on caspase inhibition. Otol. Neurotol. 28, 417–425.
Cunningham, L.L., Brandon, C.S., 2006. Heat shock inhibits both aminoglycoside- and cisplatin-induced sensory hair cell death. J. Assoc. Res. Otolaryngol. 7, 299–307.
Ding, D., Allman, B.L., Salvi, R., 2012. Review: ototoxic characteristics of platinum antitumor drugs. Anat. Rec. 295, 1851–1867.
Dyhrfjeld-Johnsen, J., Gaboyard-Niay, S., Broussy, A., Saleur, A., Brugeaud, A., Chabbert, C., 2013. Ondansetron reduces lasting vestibular deficits in a model of severe peripheral excitotoxic injury. J. Vestib. Res. 23, 177–186.
Flores-Otero, J., Xue, H.Z., Davis, R.L., 2007. Reciprocal regulation of presynaptic and postsynaptic proteins in bipolar spiral ganglion neurons by neurotrophins. J. Neurosci. 27, 14023–14034.
Gaboyard-Niay, S., Travo, C., Saleur, A., Broussy, A., Brugeaud, A., Chabbert, C., 2016. Correlation between afferent rearrangements and behavioral deficits after local excitotoxic insult in the mammalian vestibule: a rat model of vertigo symptoms. Dis. Model. Mech. 9, 1181–1192.
Golub, J.S., Tong, L., Ngyuen, T.B., Hume, C.R., Palmiter, R.D., Rubel, E.W., Stone, J.S., 2012. Hair cell replacement in adult mouse utricles after targeted ablation of hair cells with diphtheria toxin. J. Neurosci. 32, 15093–15105.
Goycoolea, M.V., Lundman, L., 1997. Round window membrane. Structure function and permeability: a review. Microsc. Res. Tech. 36, 201–211.
Grewal, S., Merchant, T., Reymond, R., McInerney, M., Hodge, C., Shearer, P., 2010. Auditory late effects of childhood cancer therapy: a report from the Children's Oncology Group. Pediatrics 125, e938–e950. doi:http://dx.doi.org/10.1542/peds.2009-1597.

Gurney, J.G., Tersak, J.M., Ness, K.K., Landier, W., Matthay, K.K., Schmidt, M.L., Children's Oncology Group, 2007. Hearing loss, quality of life, and academic problems in long-term neuroblastoma survivors: a report from the Children's Oncology Group. Pediatrics 120, e1229–e1236. doi:http://dx.doi.org/10.1542/peds2007-0178.

He, J., Yin, S., Wang, J., Ding, D., Jiang, H., 2009. Effectiveness of different approaches for establishing cisplatin-induced cochlear lesions in mice. Acta Otolaryngol. 129, 1359–1367.

Janning, M.H., Whitworth, C.A., Rybak, L.P., 1998. Experimental model of cisplatin ototoxicity in chinchillas. Otolaryngol. Head Neck Surg. 119, 574–580.

King, E.B., Salt, A.N., Kel, G.E., Eastwood, H.T., O'Leary, S.J., 2013. Gentamicin administration on the stapes footplate causes greater hearing loss and vestibulotoxicity than round window administration in guinea pigs. Hear. Res. 304, 159–166. doi:http://dx.doi.org/10.1016/j.heares.2013.07.013.

Kirkim, G., Olgun, Y., Aktas, S., Kiray, M., Kolatan, E., Altun, Z., Erçetin, P., Bagriyanik, A., Yilmaz, O., Ellidokuz, H., 2014. Is there a gender-related susceptibility for cisplatin ototoxicity? Eur. Arch. Otorhinolaryngol. 2755–2763. doi:http://dx.doi.org/10.1007/s00405-014-3283-0.

Kitsigianis, G.A., O'Leary, D.P., Davis, L.L., 1988. Active head-movement analysis of cisplatin-induced vestibulotoxicity. Otolaryngol. Head Neck Surg. 98, 82–87.

Kobayashi, H., Ohashi, N., Watanabe, Y., Mizukoshi, K., 1987. Clinical features of cisplatin vestibulotoxicity and hearing loss. ORL J. Otorhinolaryngol. Relat. Spec. 49, 67–72.

Korver, K.D.D., Rybak, L.P.P., Whitworth, C., Campbell, K.M., 2002. Round window application of D-methionine provides complete cisplatin otoprotection. Otolaryngol. Head Neck Surg. 126, 683–689. doi:http://dx.doi.org/10.1067/mhn.2002.125299.

Langer, T., am Zehnhoff-Dinnesen, A., Radtke, S., Meitert, J., Zolk, O., 2013. Understanding platinum-induced ototoxicity. Trends Pharmacol. Sci. 34, 458–469. doi:http://dx.doi.org/10.1016/j.tips.2013.05.006.

Laurell, G., Bagger-Sjöbäck, D., 1991. Dose-dependent inner ear changes after i.v. administration of cisplatin. J. Otolaryngol. 20, 158–167.

Li, Y., Ding, D., Jiang, H., Fu, Y., Salvi, R., 2011. Co-administration of cisplatin and furosemide causes rapid and massive loss of cochlear hair cells in mice. Neurotox. Res. 20, 307–319.

Llorens, J., Rodríguez-Farré, E., 1997. Comparison of behavioral, vestibular, and axonal effects of subchronic IDPN in the rat. Neurotoxicol. Teratol. 19, 117–127.

Llorens, J., Demêmes, D., Sans, A., 1993. The behavioral syndrome caused by 3,3′-iminodipropionitrile and related nitriles in the rat is associated with degeneration of the vestibular sensory hair cells. Toxicol. Appl. Pharmacol. 123, 199–210. doi:http://dx.doi.org/10.1006/taap.1993.1238.

Lo, W., Chang, C., Liao, L., Wang, C.T., Young, Y.H., Chang, Y.L., Cheng, P.W., 2015. Assessment of D-methionine protecting cisplatin-induced otolith toxicity by vestibular-evoked myogenic potential tests, ATPase activities and oxidative state in guinea pigs. Neurotoxicol. Teratol. 51, 12–20.

Madasu, R., Ruckenstein, M.J., Leake, F., Steere, E., Robbins, K.T., 1997. Ototoxic effects of supradose cisplatin with sodium thiosulfate neutralization in patients with head and neck cancer. Arch. Otolaryngol. Head Neck Surg. 123, 978–981.

Madias, N.E., Harrington, J.T., 1978. Platinum nephrotoxicity. Am. J. Med. 65, 307–314.

Miller, M.W., Agrawal, Y., 2014. Intratympanic Therapies for Menière's disease. Curr. Otorhinolaryngol. Rep. 2, 137–143.

Minami, S.B., Sha, S.-H., Schacht, J., 2004. Antioxidant protection in a new animal model of cisplatin-induced ototoxicity. Hear. Res. 198, 137–143. doi:http://dx.doi.org/10.1016/j.heares.2004.07.016.

Mukherjea, D., Jajoo, S., Sheehan, K., Kaur, T., Sheth, S., Bunch, J., Perro, C., Rybak, L.P., Ramkumar, V., 2011. NOX3 NADPH oxidase couples transient receptor potential vanilloid 1 to signal transducer and activator of transcription 1-mediated inflammation and hearing loss. Antioxid. Redox Signal. 14, 999–1010. doi:http://dx.doi.org/10.1089/ars.2010.3497.

Myers, S.F., Blakley, B.W., Schwan, S., 1993. Is cis-platinum vestibulotoxic? Otolaryngol. Head Neck Surg. 108, 322–328.

Nair, A.B., Jacob, S., 2016. Simple practice guide for dose conversion between animals and human. J. Basic Clin. Pharm. 7, 27–31.

Nakayama, M., Riggs, L.C., Matz, G.J., 1996. Quantitative study of vestibulotoxicity induced by gentamicin or cisplatin in the guinea pig. Laryngoscope 106, 162–167.

Plontke, S.K., Biegner, T., Kammerer, B., Delabar, U., Salt, A.N., 2008. Dexamethasone concentration gradients along scala tympani after application to the round window membrane. Otol. Neurotol. 29, 401–406. doi:http://dx.doi.org/10.1097/MAO.0b013e318161aaae.

Prim, M.P., de Diego, J.I., de Sarria, M.J., Gavilan, J., 2001. Vestibular and oculomotor changes in subjects treated with cisplatin. Acta Otorrinolaringol. Esp. 52, 367–370.

Rúa, F., Buffard, M., Sedó-Cabezón, L., Hernández-Mir, G., de la Torre, A., Saldaña-Ruíz, S., Chabbert, C., Bayona, J.M., Messeguer, A., Llorens, J., 2013. Vestibulotoxic properties of potential metabolites of allylnitrile. Toxicol. Sci. 135, 182–192.

Ruggiero, A., Trombatore, G., Triarico, S., Arena, R., Ferrara, P., Scalzone, M., Pierri, F., Riccardi, R., 2013. Platinum compounds in children with cancer: toxicity and clinical management. Anticancer Drugs 24, 1007–1019. doi:http://dx.doi.org/10.1097/CAD.0b013e3283650bda.

Rybak, L.P., 2007. Mechanisms of cisplatin ototoxicity and progress in otoprotection. Curr. Opin. Otolaryngol. Head Neck Surg. 15, 364–369. doi:http://dx.doi.org/10.1097/MOO.0b013e3282eee452.

Saldaña-Ruíz, S., Boadas-Vaello, P., Sedó-Cabezón, L., Llorens, J., 2013. Reduced systemic toxicity and preserved vestibular toxicity following co-treatment with nitriles and CYP2E1 inhibitors: a mouse model for hair cell loss. J. Assoc. Res. Otolaryngol. 14, 661–671.

Salt, A.N., Plontke, S.K., 2009. Principles of local drug delivery to the inner ear. Audiol. Neurootol. 14, 350–360. doi:http://dx.doi.org/10.1159/000241892.

Schacht, J., Talaska, A.E., Rybak, L.P., 2012. Cisplatin and aminoglycoside antibiotics: hearing loss and its prevention. Anat. Rec. (Hoboken) 295, 1837–1850. doi:http://dx.doi.org/10.1002/ar.22578.

Schaefer, S.D., Wright, C.G., Post, J.D., Frenkel, E.P., 1981. Cis-platinum vestibular toxicity. Cancer 47, 857–859.

Schmitt, N.C., Rubel, E.W., Nathanson, N.M., 2009. Cisplatin-induced hair cell death requires STAT1 and is attenuated by epigallocatechin gallate. J. Neurosci. 29, 3843–3851.

Schweitzer, V.G., Rarey, K.E., Dolan, D.F., Abrams, G.E., Sheridan, C., 1986. Vestibular morphological analysis of the effects of cisplatin vs. platinum analogs, CBDCA (JM-8) and CHIP (JM-9). Laryngoscope 96, 959–974.

Seoane, A., Demêmes, D., Llorens, J., 2003. Distal effects in a model of proximal axonopathy: 3,3′-iminodipropionitrile causes specific loss of neurofilaments in rat vestibular afferent endings. Acta Neuropathol. 106, 458–470.

Sera, K., Harada, Y., Tagashira, N., Suzuki, M., Hirakawa, K., Ohya, T., 1987. Morphological changes in the vestibular epithelia and ganglion induced by ototoxic drug. Scanning Microsc. 1, 1191–1197.

Sergi, B., Ferraresi, A., Troiani, D., Paludetti, G., Fetoni, A.R., 2003. Cisplatin ototoxicity in the guinea pig: vestibular and cochlear damage. Hear. Res. 182, 56–64. doi:http://dx.doi.org/10.1016/S0378-5955(03)00142-4.

Soler-Martín, C., Boadas-Vaello, P., Verdú, E., Garcia, N., Llorens, J., 2014. Chronic proximal axonopathy in rats is associated with long-standing neurofilament depletion in neuromuscular junctions and behavioral deficits. J. Neuropathol. Exp. Neurol. 73, 568–579.

Strauss, M., Towfighi, J., Lord, S., Lipton, A., Harvey, H.A., Brown, B., 1983. Cis-platinum ototoxicity: clinical experience and temporal bone histopathology. Laryngoscope 93, 1554–1559.

Tian, C.J., Kim, Y.J., Kim, S.W., Lim, H.J., Kim, Y.S., Choung, Y.H., 2013. A combination of cilostazol and Ginkgo biloba extract protects against cisplatin-induced Cochleo-vestibular dysfunction by inhibiting the mitochondrial apoptotic and ERK pathways. Cell. Death Dis. 4, e509. doi:http://dx.doi.org/10.1038/cddis.2013.33.

Tofthagen, C., Overcash, J., Kip, K., 2012. Falls in persons with chemotherapy-induced peripheral neuropathy. Support. Care Cancer 20, 583–589. doi:http://dx.doi.org/10.1007/s00520-011-1127-7.

Vignaux, G., Chabbert, C., Gaboyard-Niay, S., Travo, C., Machado, M.L., Denise, P., Comoz, F., Hitier, M., Landemore, G., Philoxène, B., Besnard, S., 2012. Evaluation of the chemical model of vestibular lesions induced by arsanilate in rats. Toxicol. Appl. Pharmacol. 258, 61–71. doi:http://dx.doi.org/10.1016/j.taap.2011.10.008.

Wang, J., Lloyd Faulconbridge, R.V., Fetoni, A., Guitton, M.J., Pujol, R., Puel, J.L., 2003. Local application of sodium thiosulfate prevents cisplatin-induced hearing loss in the guinea pig. Neuropharmacology 45, 380–393 (S002839080300).

Whitworth, C.A., Ramkumar, V., Jones, B., Tsukasaki, N., Rybak, L.P., 2004. Protection against cisplatin ototoxicity by adenosine agonists. Biochem. Pharmacol. 67, 1801–1807. doi:http://dx.doi.org/10.1016/j.bcp.2004.01.010.

Wright, C.G., Schaefer, S.D., 1982. Inner ear histopathology in patients treated with cis-platinum. Laryngoscope 92, 1408–1413.

Xia, L., Chen, Z., Yin, S., 2012. Ototoxicity of cisplatin administered to guinea pigs via the round window membrane. J. Toxicol. Sci. 37, 823–830.

Zhang, M., Liu, W., Ding, D., Salvi, R., 2003. Pifithrin-α supresses p53 and protects cochlear and vestibular hair cells from cisplatin-induced apoptosis. Neuroscience 120, 191–205. doi:http://dx.doi.org/10.1016/S0306-4522(03)00286-0.

Zou, J., Pyykkö, I., 2013. Enhanced oval window and blocked round window passages for middle-inner ear transportation of gadolinium in guinea pigs with a perforated round window membrane. Eur. Arch. Otorhinolaryngol. doi:http://dx.doi.org/10.1007/s00405-013-2856-7.