Research Article

Reduced Systemic Toxicity and Preserved Vestibular Toxicity Following Co-treatment with Nitriles and CYP2E1 Inhibitors: a Mouse Model for Hair Cell Loss

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ABSTRACT

Several nitriles, including allylnitrile and *cis*-crotononitrile, have been shown to be ototoxic and cause hair cell degeneration in the auditory and vestibular sensory epithelia of mice. However, these nitriles can also be lethal due in large part to the microsomal metabolic release of cyanide, which is mostly dependent on the activity of the 2E1 isoform of the cytochrome P450 (CYP2E1). In this study, we co-administered mice with a nitrile and, to reduce their lethal effects, a selective CYP2E1 inhibitor: diallylsulfide (DAS) or *trans*-1,2-dichloroethylene (TDCE). Both in female 129S1/SvImJ (129S1) mice co-treated with DAS and *cis*-crotononitrile and in male RjOrl:Swiss/CD-1 (Swiss) mice co-treated with TDCE and allylnitrile, the nitrile caused a dose-dependent loss of vestibular function, as assessed by a specific behavioral test battery, and of hair cells, as assessed by hair bundle counts using scanning electron microscopy. In the experiments, the CYP2E1 inhibitors provided significant protection against the lethal effects of the nitriles and did not diminish the vestibular toxicity as assessed by behavioral effects in comparison to

animals receiving no inhibitor. Additional experiments using a single dose of allylnitrile demonstrated that TDCE does not cause hair cell loss on its own and does not modify the vestibular toxicity of the nitrile in either male or female 129S1 mice. In all the experiments, high vestibular dysfunction scores in the behavioral test battery predicted extensive to complete loss of hair cells in the utricles. This provides a means of selecting animals for subsequent studies of vestibular hair cell regeneration or replacement.

Keywords: ototoxicity, vestibular toxicity, allylnitrile, *cis*-crotononitrile, mouse, hair cell ablation

INTRODUCTION

The sensory epithelia of the mammalian inner ear have null or limited capacity for regeneration of the mechanotransducer hair cells. Thus, permanent auditory and balance deficits may result from hair cell loss due to a variety of causes, including genetic factors, aging, noise, head trauma, and exposure to ototoxic substances. Nowadays, significant research efforts are directed at replacing the lost hair cells through cell implant strategies or stimulation of hair cell regeneration in damaged ears. Prophylactic treatments that can prevent hair cell degeneration are also a focus of interest. These studies require dependable animal models of hair cell loss and,

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particularly, tend to use mice models, owing to the availability of probes, antibodies, and genetic tools for this species.

In several species, such as the guinea pig, hair cell degeneration is commonly achieved by exposing animals to aminoglycoside antibiotics, a group of compounds that are well known for their ototoxic effects in humans (Forge and Schacht 2000). However, mice are particularly resistant to hair cell degeneration induced by systemic aminoglycoside administration. For instance, a recent study aimed at refining ototoxicity models in the mouse found it necessary to dose mice with intramuscular kanamycin every 12 h for 15 days to obtain significant auditory loss with moderate (30 %) mortality, while even less favorable outcomes were obtained with gentamycin, amikacin, and neomycin (Murillo-Cuesta et al. 2009). Extensive ototoxic damage with limited systemic toxicity can be achieved by intratympanic aminoglycoside administration (Heydt et al. 2004; Ishibashi et al. 2009), but this mode of administration involves surgery or a delicate trans-tympanic administration that often results in highly variable lesions. To reliably produce acute cochlear lesions, potentiation of kanamycin ototoxicity using the loop diuretics furosemide (Oesterle et al. 2008) or bumetanide (Taylor et al. 2008) has been successful. However, this approach offers limited flexibility for cochlear studies and leaves the vestibular system totally unaffected.

Over the last 20 years, several nitriles, compounds containing cyano (R-CN) groups, have been shown to be ototoxic in animal models. Hair cell loss has been found in the vestibular and auditory epithelia of rats and mice after exposure to 3,3'-iminodipropionitrile (IDPN) (Llorens et al. 1993; Llorens and Demêmes 1994; Crofton et al. 1994), allylnitrile (Balbuena and Llorens 2001; Gagnaire et al. 2001), crotonitrile (Gagnaire et al. 2001; Llorens et al. 1998), and *cis*-2-pentenitrile (Gagnaire et al. 2001; Saldaña-Ruiz et al. 2012a, b). In the case of crotonitrile, only the *cis*-isomer is ototoxic in rats and mice (Balbuena and Llorens 2003; Soler-Martín et al. 2007), while *trans*-crotonitrile is ototoxic at sublethal levels in mice (Saldaña-Ruiz et al. 2012a) but not in rats (Boadas-Vaello et al. 2005). The ototoxic properties of nitriles have many features in common with those of aminoglycoside antibiotics. Like the antibiotics, the ototoxic nitriles affect both the auditory and the vestibular system (Llorens et al. 1993; Crofton et al. 1994; Balbuena and Llorens 2001; Soler-Martín et al. 2007) and show selectivity for hair cells as the primary target (Llorens and Demêmes 1994). Another similar feature is the susceptibility of multiple mammalian and non-mammalian species (Soler-Martín et al. 2007). Finally, the nitriles also resemble the antibiotics in the characteristic intra-epithelial and inter-epithelial differences in susceptibility: the hair cell loss

progresses in a basal to apical order in the cochlea, in a central to peripheral order within each vestibular epithelia, and in a crista>utricle>sacculle order across the vestibular epithelia (Llorens et al. 1993; Llorens and Demêmes 1994; Balbuena and Llorens 2001, 2003; Soler-Martín et al. 2007). Thus, nitriles may be a good tool for causing hair cell degeneration in the mouse auditory and vestibular epithelia.

Among the ototoxic nitriles, IDPN is a reliable and easy-to-use tool for hair cell lesioning. IDPN offers a wide window of ototoxic doses with no associated mortality (Llorens et al. 1993; Llorens and Demêmes 1994), great flexibility in effective dosing regimens (Llorens and Rodríguez-Farré 1997; Seoane et al. 2001a, b), and can be used across different species, including rats, mice, guinea pigs, and frogs (Soler-Martín et al. 2007). IDPN has been used to achieve vestibular lesions in some functional or regeneration studies (Brueckner et al. 1999; Khan et al. 2004; Brugeaud et al. 2007; Schlecker et al. 2011). However, the use of IDPN as a model vestibular toxin is sometimes hampered by the concurrence of its well-known toxic effect on neurofilament transport. IDPN causes a reduction in the axonal neurofilament transport rate (Griffin et al. 1978), which is associated with axonal swelling in the proximal internodal segments of large myelinated axons (Chou and Hartmann 1964), and neurofilament accumulation in the vestibular ganglion neuronal perikarya (Llorens and Demêmes 1996).

Unlike IDPN, allylnitrile and *cis*-crotonitrile seem to have little or no potential to cause neurofilamentous axonopathy (Llorens et al. 1998; Soler-Martín et al. 2011). However, their high acute toxicity often leads to mortality and makes them unsuitable as selective ototoxic tools. We have recently reported that the acute toxicity of these two nitriles is largely due to cyanide release from the parent nitriles through metabolism by the 2E1 isoform of cytochrome P450 (CYP2E1), as demonstrated by reduced mortality in CYP2E1-null mice. In contrast, the vestibular toxicity of these compounds remains unchanged in CYP2E1-null mice (Boadas-Vaello et al. 2007, 2009; Saldaña-Ruiz et al. 2012a). As control experiments in those studies, we hypothesized that pharmacological inhibition of CYP2E1 in wild-type mice would reduce the acute toxicity of these nitriles, but not their vestibular toxicity. Accordingly, we obtained behavioral evidence demonstrating the hypothesized effect in mice exposed to allylnitrile and co-treated with the CYP2E1 inhibitor diallylsulfide (DAS) (Boadas-Vaello et al. 2009). The aim of the present work is to extend those preliminary data via a complete behavioral and histological assessment, thus paving the way for the use of these nitriles in studies requiring hair cell ablation in mice. To this end, we performed four experiments to test the protection from

acute toxicity and the loss of vestibular function and hair cells and to establish the relationship between the functional and histological alterations. Because sex differences have been found in nitrile metabolism and toxicity in mice (Chanas et al. 2003; Boadas-Vaello et al. 2007; Saldaña-Ruiz et al. 2012a), only one sex was used in each of the experiments reported here. Vestibular lesions were successfully obtained in both sexes and also in two different strains of mice.

METHODS

Chemicals and reagents

Allylnitrile (>98 %) was purchased from Merck-Schuchard (Hohenbrunn bei München, Germany) and DAS (97 %) and *trans*-1,2-dichloroethylene (98 %) (TDCE) from Sigma-Aldrich Química S.A. (Madrid, Spain). *cis*-Crotononitrile was obtained by fractional distillation from a commercially available racemic mixture (99 %, *cis/trans* ratio of approximately 60:40, Aldrich Química, Alcobendas, Spain), as previously described (Balbuena and Llorens 2003). Fractions with an isomeric purity greater than 97 % (by $[1H]$ -NMR, 300 MHz, using $CDCl_3$ as the solvent in a Varian Unity 300 spectrometer) were used in the present series of experiments. Other chemicals were of analytical grade, as obtained from common commercial sources.

Animals

Animal care and use were in accordance with Acts 5/1995 and 214/1997 of the regional authorities ("Generalitat") of Catalonia and approved by the Commission on Animal Experimentation. Two different strains were evaluated, and only one sex was used in each of the experiments. In one experiment, we used male RjOrl:Swiss/CD-1 (Swiss) mice purchased from Janvier (Le-Genest-Saint-Isle, France). In other experiments, we used male or female 129S1/SvImJ (129S1) mice from a local colony established by breeding pairs obtained from the Jackson Laboratory (Bar Harbor, ME, USA). Mice were housed two to four per cage in standard Macrolon cages (28×28×15 cm) with wood shavings as bedding. They were given standard diet pellets (Harlan Teklad Global Diet 2014) ad libitum. For the histological study, mice were anesthetized with 400 mg kg⁻¹ chloral hydrate and decapitated. The temporal bones were then directly immersed in cold fixative solution for immediate dissection of the vestibular sensory epithelia.

Dosing and experimental protocol

Allylnitrile and *cis*-crotononitrile were administered p.o. in 6 ml/kg of corn oil. Nitrile dose selection was

based on our previous data (Boadas-Vaello et al. 2007, 2009; Saldaña-Ruiz et al. 2012a). The selective CYP2E1 inhibitor DAS (Brady et al. 1991) was administered p.o. in 6 ml/kg of corn oil; DAS acts as a competitive inhibitor of CYP2E1 and is also metabolized to suicide inhibitors of the same enzyme. Another suicide inhibitor selective for CYP2E1, TDCE (Lilly et al. 1998; Mathews et al. 1998), was administered i.p. in 6 ml/kg of corn oil.

Initially, two dose-response experiments were run in three parts each, with two to four animals per treatment condition in each part. Then, a further two experiments using a single dose of the nitrile were run. In the first dose-response experiment, female 129S1 mice received 0, 1.75, 2.25, 2.75, or 3.25 mmol/kg of *cis*-crotononitrile 2 h after administration of DAS at 0 (corn oil, vehicle control) or 200 mg/kg. In contrast to our previous study (Boadas-Vaello et al. 2009), only one dose of DAS was administered, because the use of three doses had been observed to cause significant toxic effects. In the 3.25+ oil group, only the four animals in the first part of the experiment were dosed because of the high mortality recorded among them.

In the second dose-response experiment, male Swiss mice received 0, 1.0, 1.25, or 1.5 mmol/kg of allylnitrile and TDCE at 0 (corn oil, vehicle control) or 100 mg/kg, administered 30 min before and 6 and 24 h after administration of allylnitrile. The use of three doses was chosen to ensure the maximal effect of TDCE over the time period of allylnitrile ototoxicity, according to data in the literature (Mathews et al. 1998).

In the third experiment (the first of the two single-dose experiments), male 129S1 mice received 0 or 1.0 mmol/kg of allylnitrile and 3× TDCE at 0 or 100 mg/kg at the same times as above. In the fourth and last experiment, female 129S1 mice received 0 or 1.5 mmol/kg of allylnitrile and 3× TDCE at 0 or 100 mg/kg.

The animals were observed daily for 3–4 days and at least twice a week afterwards and rated for overall toxicity. The animals were killed if they met the criteria of the ethical limits of suffering. Animals that were killed were considered for data analysis in the same way as those that died spontaneously. Vestibular dysfunction was assessed 2–3, 7, and 21 days after nitrile exposure, and histological analysis was performed 4–5 weeks after dosing. In our experience (Llorens et al. 1993; Balbuena and Llorens 2001, 2003; Soler-Martín et al. 2007; Boadas-Vaello et al. 2007, 2009), no significant change in scores of vestibular dysfunction occurs between 3 and 4 weeks post-dosing in rats and mice with high scores that reflect severe dysfunction.

Behavioral analysis of vestibular function

The alteration of vestibular function was determined using a battery of behavioral tests, as previously

reported (Soler-Martín et al. 2007; Boadas-Vaello et al. 2007, 2009; Saldaña-Ruiz et al. 2012a). The same experimenter was responsible for dosing and behavioral analysis. However, animals were assigned to group treatments in a pseudorandom order and the rating sheets did not contain treatment information, so the experimenter performed the behavioral assessment under blind conditions. Briefly, the mice were placed for 1 min in an open arena (a rat cage) and the experimenter rated the animals from 0 (normal behavior) to 4 (maximal deficit in behavior) for circling, retropulsion, and abnormal head movements. Circling was defined as stereotyped circulatory ambulation. Retropulsion consisted of backward movement. Abnormal head movements consisted of intermittent extreme backward extension of the neck. The mice were then rated from 0 to 4 for the tail hang reflex, contact inhibition of the righting reflex, and air righting reflex tests. When lifted by the tail, normal mice exhibit a “landing” response consisting of forelimb extension. Mice with impaired vestibular function bend ventrally, sometimes “crawling” up towards their tails, thus tending towards occipital landing. For contact inhibition of the righting reflex, mice were flipped supine on a horizontal surface and a rigid plastic board was lightly placed in contact with the soles of their feet. Healthy mice quickly right themselves, but vestibular-deficient mice lie on their back, with their feet up and “walk” with respect to the ventral surface. For the air righting reflex, the animals were dropped supine from a height of 10 cm onto a foam cushion. Normal mice are able to right themselves in the air, whereas vestibular-deficient mice are not. A summary score of vestibular dysfunction was obtained by adding up the scores for all the behavior patterns.

Histology

We examined surface preparations of the vestibular sensory epithelia using scanning electron microscopy (SEM), following standard procedures as described elsewhere (Soler-Martín et al. 2007; Boadas-Vaello et al. 2007, 2009; Saldaña-Ruiz et al. 2012a). Briefly, the temporal bones were obtained and immediately immersed in ice-cold 2.5 % glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2), and the sensory vestibular epithelia were quickly dissected. The samples were fixed for 1.5 h in the same solution, post-fixed for 1 h in 1 % osmium tetroxide in cacodylate buffer, dehydrated with increasing concentrations of ethanol up to 100 %, and dried in a critical point dryer using liquid CO₂. The dried sensory epithelia were coated with gold and observed in a Quanta-200 SEM (FEI Company, Hillsboro, OR, USA). To evaluate hair cell loss, hair bundles were counted in representative

images of the central part of the utricle with the assistance of ImageJ software (National Institute of Mental Health, Bethesda, MD, USA). In optimally preserved utricles, complete counts were obtained from images of large areas such as those shown in Figure 9, but smaller areas, such as those shown in Figure 4, were used if the larger ones included parts that could not be counted because the utricle was damaged or was partially covered with the otoconial membrane or debris.

Statistics

Mortality data are shown as the percentage of dead animals. The effect of DAS or TDCE on the survival of mice exposed to high doses of the nitriles was analyzed by Kaplan–Meier analysis using the Breslow statistic. Behavioral data are shown as mean $\pm$ standard error of raw scores of vestibular dysfunction. These data were tested with repeated measures MANOVA (Wilks’ criterion) with the day as the within-subject factor. Orthogonal contrasts followed by Duncan’s test were used for post hoc analysis. Hair cell bundle counts were normalized as the percentage of the mean control value in the same experiment, to facilitate comparisons across experiments, and are shown as mean $\pm$ standard error. These data were analyzed by one-way ANOVA followed by Duncan’s test for post hoc analysis. In all analyses, the PASW 18.0 for Windows software package was used.

RESULTS

Dose-dependent effects of *cis*-crotononitrile and DAS on female 129S1 mice

Female 129S1 mice exposed to *cis*-crotononitrile displayed acute toxicity, including weight loss and death (Fig. 1). Co-treatment with DAS partially reduced this acute toxicity. Thus, survival after 2.75 mmol/kg of *cis*-crotononitrile was increased from three out of eight to seven out of eight by DAS co-treatment ($\chi^2_{(1)}=5.184$, $P=0.023$). However, only partial protection was afforded, since similar high mortality rates were observed after 3.25 mmol/kg of *cis*-crotononitrile both in groups of animals co-treated with DAS and those that were not. The decrease in acute toxicity was also observed in the body weight data, which showed a maximal reduction of the mean group value of 18 % after the high *cis*-crotononitrile dose without DAS co-treatment and of 13 % when the same dose of the nitrile was given with DAS co-treatment (data not shown).

cis-Crotononitrile caused a dose-dependent increase in the scores of vestibular dysfunction in female 129S1 mice whether co-treated with DAS or not

FIG. 1. Effect of co-treatment with the CYP2E1 inhibitor, diallylsulfide (DAS), on the acute toxicity of *cis*-crotononitrile (cCN) in 129S1 female mice.

(Fig. 2). The surviving animals in the 3.25 mmol/kg of *cis*-crotononitrile groups showed high scores (>17), but were excluded from the analysis due to the small group size. In the remaining groups, an analysis of the test battery data indicated significant effects of day, $F_{(3, 48)}=19.3$, $P=0.000$; day by treatment, $F_{(21, 138)}=2.5$, $P=0.001$; and treatment, $F_{(7, 50)}=8.5$, $P=0.000$. Significant group differences were detected at all the post-dosing experimental times, all $F_{(7, 56)}>6.8$, $P_s=0.000$. The 1.75 mmol/kg of *cis*-crotononitrile groups showed no differences from controls, but doses of 2.25 and 2.75 mmol/kg induced a significant loss of vestibular function, which was larger in the groups receiving the highest dose. Animals co-treated with DAS did not have lower scores than the animals receiving no co-treatment in any case.

The vestibular sensory epithelia of 18 animals co-treated with *cis*-crotononitrile and DAS were examined by SEM at 4–5 weeks after dosing. Control animals and

FIG. 2. Effects of *cis*-crotononitrile (0, 1.75, 2.25, and 2.75 mmol/kg) on the vestibular function test in 129S1 female mice with (red symbols) or without (yellow symbols) co-treatment with diallylsulfide (DAS). Numbers in parentheses are the number of animals. Data are the mean group vestibular rating scores $\pm$ standard error. The maximum score is 24. a–c Groups not sharing a letter are significantly different, $P<0.05$, by Duncan's test.

animals administered with *cis*-crotononitrile that exhibited no alterations in behavior showed dense hair bundles from the sensory cells in the vestibular epithelia (Fig. 3A, B), with little or no difference from descriptions in the literature of control vestibular sensory epithelia (Csillag 2005; Soler-Martín et al. 2007). Hair bundle loss was observed in animals exposed to *cis*-crotononitrile with increased rating scores of vestibular dysfunction (Fig. 3C–F). Two animals

FIG. 3. Effects of *cis*-crotononitrile (1.75, 2.25, 2.75, and 3.25 mmol/kg) on the vestibular sensory epithelia of female 129S1 mice co-treated with diallylsulfide (DAS), as observed by SEM 4–5 weeks after exposure. **A** Crista of a mouse treated with 1.75 mmol/kg of *cis*-crotononitrile displaying no evidence of vestibular dysfunction (vestibular rating scores at days 7 and 21 were 1). Minor evidence of damage includes the lack of a few hair bundles (black arrow) and presence of a few coalescent ciliary bundles (white arrow) in the apical part of the crista. However, the density of hair bundles and overall appearance of the sensory epithelium were similar to those of control mice. **B** Utricle of the same mouse as in **A**. Note the regular distribution of hair bundles over the surface of the sensory epithelium. **C** Crista of a mouse treated with 2.75 mmol/kg of *cis*-crotononitrile displaying moderate loss of vestibular function (vestibular rating score at day 21 was 7). Note the extensive loss of hair cell bundles. **D** Utricle of the same mouse as in **C**, displaying evident loss of hair cell bundles. **E** Crista of a mouse treated with 2.75 mmol/kg of *cis*-crotononitrile, displaying major loss of vestibular function (vestibular rating score at day 21 was 19). Virtually, no hair cell bundles remain in the epithelium. **F** Utricle of another mouse treated with 2.75 mmol/kg of *cis*-crotononitrile, displaying major loss of vestibular function (vestibular rating score at day 21 was 18). Virtually, no hair cell bundles remain in the epithelium, which became more fragile and split into two halves during final manipulation. Scale bars=100 μ m in **A**, **C**, and **E**; 200 μ m in **B**, **D**, and **F**.

displayed moderate vestibular rating scores (6 and 7 at day 7) and moderate hair cell loss (Fig. 3C, D), while most showed no obvious behavioral and histological effect or nearly complete hair cell loss (Fig. 3E, F) in association with high (≥ 15) vestibular rating scores. Figure 4 shows the relationship between mean group behavioral scores at day 21 and mean group hair bundle counts.

Dose-dependent effects of allylnitrile and TDCE on male Swiss mice

TDCE reduced the acute toxicity of allylnitrile in male Swiss mice (Fig. 5). Thus, the mortality after 1.25 mmol/kg of allylnitrile was four out of eight with no TDCE co-treatment and 1/11 in co-treated animals ($\chi^2_{(1)}=3.134$, $P=0.077$). Based on previously published observations (Boadas-Vaello et al. 2009), we predicted excessive lethality after 1.50 mmol/kg of allylnitrile without TDCE co-treatment. However, we explored the response of the animals to this dose of allylnitrile with TDCE co-treatment. None of the four animals receiving 1.50 mmol/kg of allylnitrile and TDCE died or reached the ethical limits of suffering. These animals showed the greatest body weight effects, a moderate 10 % drop in body weight recorded at day 2 after dosing. Animals that received 1.25 mmol/kg of allylnitrile showed a maximal drop in body weight of only 5 % at day 1 after dosing (data not shown).

Allylnitrile caused a dose-dependent increase in scores of vestibular dysfunction in male Swiss mice whether co-treated with TDCE or not (Fig. 6). Analysis of the test battery data indicated significant effects of day, $F_{(3, 37)}=48.8$, $P=0.000$; day by treatment, $F_{(18, 105)}=4.2$, $P=0.000$; and treatment,

FIG. 4. Effects of *cis*-crotononitrile (0, 1.75, 2.25, 2.75, and 3.25 mmol/kg) on vestibular function and vestibular morphology in female 129S1 mice co-treated with diallylsulfide (DAS). Relationship between behavioral rating scores at day 21 and hair cell counts in the utricle. a–c Groups not sharing a letter are significantly different, $P<0.05$, by Duncan's test, for histological (above the symbol) and behavioral (on the left of the symbol) data.

FIG. 5. Effect of co-treatment with the CYP2E1-inhibitor, *trans*-1,2-dichloroethylene (TDCE), on the acute toxicity of allylnitrile (AllylCN) in male Swiss mice. N.D. and 0/4: not determined, no animals were dosed with 1.5 mmol/kg without TDCE co-treatment; and dead/total at the same dose with TDCE co-treatment.

$F_{(6, 39)}=16.0$, $P=0.000$. Significant group differences were detected at all the post-dosing experimental times, all $F_{s(6, 39)}>11.8$, $P_s=0.000$.

The vestibular sensory epithelia of 14 animals co-treated with TDCE and allylnitrile were examined by SEM 4–5 weeks after dosing. Control animals and animals administered with allylnitrile that had no alterations in behavior showed dense hair bundles from the sensory cells in the vestibular epithelia (Fig. 7A, B), as previously observed with the 129S1 female mice. However, one animal with control-like behavior in the test battery showed a noticeable loss of hair bundles in the apical region of the crista (Fig. 7C). Extensive to complete hair cell loss in cristas and utricles (Fig. 7E–H) was associated with high (≥ 12) scores

FIG. 6. Effects of allylnitrile (0, 1.0, 1.25, and 1.5 mmol/kg) on the vestibular function test in Swiss male mice, with (red symbols) or without (yellow symbols) co-treatment with *trans*-1,2-dichloroethylene (TDCE). Numbers in parentheses are the number of animals. Data are the mean group vestibular rating scores $\pm$ standard error. The maximum score is 24. a–c Groups not sharing a letter are significantly different, $P<0.05$, by Duncan's test.

FIG. 7. Effects of allylnitrile (1.0, 1.25, and 1.5 mmol/kg) on the vestibular sensory epithelia of male Swiss mice co-treated with *trans*-1,2-dichloroethylene (TDCE), as observed by SEM 4–5 weeks after exposure. **A, B** Crista and utricle of a mouse treated with control vehicles and displaying no evidence of vestibular dysfunction (vestibular rating score at day 21 was 0). **C** Crista of a mouse treated with 1.0 mmol/kg of allylnitrile displaying no obvious loss of vestibular function (vestibular rating score at day 21 was 0). Note the significant loss of hair cell bundles in the apical part of the receptor. **D** Utricle of the same mouse as in **C**, displaying only scant loss of hair cell bundles. **E** Crista of a mouse treated with 1.25 mmol/kg of allylnitrile displaying a significant loss of vestibular function (vestibular rating scores were 16 at day 3; 12 at day 7; and 2 at day 21). Note the extensive loss of hair cell bundles. **F** Utricle of another mouse treated with 1.25 mmol/kg of allylnitrile displaying a significant loss of vestibular function (vestibular rating score at day 21 was 13). Less than half of the hair cell bundles remain in the epithelium. **G, H** Crista and utricle from the same mouse treated with 1.5 mmol/kg of allylnitrile, showing high vestibular rating scores (17 at day 21) and almost complete loss of hair cell bundles. Scale bars= 50 μ m in **A, C, E**, and **G**; 30 μ m in **B, D, F**, and **H**.

of vestibular dysfunction. The relationship between behavioral scores at day 21 and hair bundle counts is shown in Figure 8.

FIG. 8. Effects of allylnitrile (0, 1.0, 1.25, and 1.50 mmol/kg) on vestibular function and vestibular morphology in male Swiss mice co-treated with *trans*-1,2-dichloroethylene (TDCE). Relationship between behavioral rating scores at day 21 and hair cell counts in the utricle. *a–c* Groups not sharing a letter are significantly different, $P<0.05$, by Duncan's test, for histological (above the symbol) and behavioral (on the left of the symbol) data.

Effects of allylnitrile and TDCE on male and female 129S1 mice

The effects of allylnitrile and TDCE co-administration were evaluated in male 129S1 mice at the single dose of 1.0 mmol/kg of allylnitrile. In the groups of animals given allylnitrile: 3 out of 15 died with no TDCE co-treatment, while zero out of nine died with TDCE co-treatment. No significant differences in body weight among treatment groups were recorded in this experiment. Allylnitrile caused an increase in scores of vestibular dysfunction, which was similar in mice co-treated with TDCE and those that were not. Analysis of the test battery data indicated significant effects of day, $F_{(3, 29)}=18.7$, $P=0.000$; day by treatment, $F_{(9, 71)}=5.0$, $P=0.000$; and treatment, $F_{(3, 31)}=13.3$, $P=0.000$. Significant group differences were detected at all the post-dosing experimental times, all $F_{(3, 31)}>10.7$, $P_s=0.000$. At all times, statistically significant differences were present between the two groups of animals treated and the two groups not treated with allylnitrile, but in no case was a difference recorded between oil (control) co-treated and TDCE co-treated animals (Duncan's test, $P<0.05$). In this experiment, all the animals in the four experimental groups were evaluated for hair cell loss by SEM. These observations demonstrated that TDCE does not cause hair cell loss and does not modify the hair cell loss caused by 1.0 mmol/kg of allylnitrile (Fig. 9). Hair cell counts showed a similar mean 60 % reduction after allylnitrile in the animals co-treated with oil or TDCE. Thus, the group differences revealed by a significant ANOVA, $F_{(3, 26)}=12.2$, $P=0.000$, resulted from the allylnitrile effect, with similar hair cell bundle counts in oil and TDCE groups (Duncan's test, $P>0.05$). The relationship between behavioral

FIG. 9. Effects of *trans*-1,2-dichloroethylene (TDCE) and allylnitrile (1.0 mmol/kg) on the vestibular sensory epithelia of male 129S1 mice as observed by SEM 4–5 weeks after exposure. **A** Control. Utricle of a mouse treated with control vehicles; vestibular rating score at day 21 was 0. **B** Control-like appearance of a utricle from a mouse exposed to TDCE but not to allylnitrile and displaying no evidence of vestibular dysfunction (vestibular rating score at day 21 was 0). **C** Utricle of a mouse treated with 1.0 mmol/kg of allylnitrile, without TDCE co-treatment, and displaying significant loss of vestibular function (vestibular rating score at day 21 was 22). **D** Utricle of a mouse co-treated with TDCE and 1.0 mmol/kg of allylnitrile and suffering significant loss of vestibular function (vestibular rating score at day 21 was 21). Note the similarity of the sensory epithelia after exposure (**B**, **D**) or not (**A**, **C**) to TDCE. Scale bars=50 μ m.

scores at day 21 and SEM counts is shown in Figure 10.

Female 129S1 mice administered 1.5 mmol/kg of allylnitrile and not co-treated with TDCE displayed a high level of mortality (11/15) which was only marginally attenuated (5/11) by TDCE co-treatment ($\chi^2_{(1)}=2.798$,

$P=0.094$). In this experiment, no significant protective effect of TDCE on the effect of the nitrile on body weight was observed; maximal loss was recorded for both allylnitrile groups at day 3 after dosing and was 18 % in the animals co-treated with oil (control) and 15 % in the animals co-treated with TDCE. Allylnitrile caused a similar increase in scores of vestibular dysfunction, in mice co-treated with TDCE and those that were not. Analysis of the test battery data indicated significant effects of day, $F_{(3, 15)}=1485$, $P=0.000$; day by treatment, $F_{(9, 37)}=43.8$, $P=0.000$; and treatment, $F_{(3, 17)}=518$, $P=0.000$. Significant group differences were detected at all the post-dosing experimental times, all $F_{s(3, 17)}>540$, $P_s=0.000$. At all times, statistically significant differences were present between the two groups of animals treated and the two groups not treated with allylnitrile, but in no case was a difference recorded between oil co-treated and TDCE co-treated animals (Duncan's test, $P<0.05$). When all animals in the experiment were examined by SEM for loss of hair cells, the data obtained demonstrated that TDCE does not cause hair cell loss and does not modify the hair cell loss caused by 1.5 mmol/kg of allylnitrile. Thus, the group differences revealed by a significant ANOVA, $F_{(3, 17)}=58.8$, $P=0.000$, resulted from the allylnitrile effect, with similar hair cell bundle counts in oil and TDCE groups (Duncan's test, $P>0.05$). The relationship between behavioral scores at day 21 and SEM counts is shown in Figure 11.

DISCUSSION

A dependable model for vestibular hair cell degeneration in rodents, and mice in particular, is a long-

FIG. 10. Effects of allylnitrile (0 and 1.0 mmol/kg) on vestibular function and vestibular morphology in male 129S1 mice co-treated or not with *trans*-1,2-dichloroethylene (TDCE). Relationship between behavioral rating scores at day 21 and hair cell counts in the utricle. *a*, *b* Groups not sharing a letter are significantly different, $P<0.05$, by Duncan's test, for histological (*above the symbol*) and behavioral (*on the left of the symbol*) data.

FIG. 11. Effects of allylnitrile (0 and 1.5 mmol/kg) on vestibular function and vestibular morphology in female 129S1 mice co-treated or not with *trans*-1,2-dichloroethylene (TDCE). Relationship between behavioral rating scores at day 21 and hair cell counts in the utricle. *a*, *b* Groups not sharing a letter are significantly different, $P<0.05$, by Duncan's test, for histological (*above the symbol*) and behavioral (*on the left of the symbol*) data.

standing need in inner ear research, for example, in studies to develop strategies for alleviating human labyrinth damage by hair cell protection, regeneration, or replacement. One possible model is exposure to the nitriles allylnitrile and *cis*-crotononitrile, with a known ototoxic potential (Balbuena and Llorens 2001, 2003; Gagnaire et al. 2001; Soler-Martín et al. 2007; Boadas-Vaello et al. 2007, 2009; Saldaña-Ruiz et al. 2012a). In this study, we have evaluated refining these models through the use of pharmacological agents that inhibit the CYP2E1 enzyme to reduce the acute systemic toxicity of the nitriles. The data obtained show that co-treatment with TDCE and a single dose of allylnitrile (1.0 or 1.25 mmol/kg) in male Swiss or 129S1 mice results in extensive hair cell loss with little or no associated mortality. Other data indicate that female mice, the alternative ototoxic nitrile *cis*-crotononitrile, and the alternative CYP2E1 inhibitor DAS can also be used. Using these models, we assessed the relationship between the extent of utricular damage and the vestibular rating scores from a test battery designed to evaluate vestibular dysfunction; the data show that the behavioral scores are predictive of the extent of the pathology and can thus be used to select animals with extensive loss of hair cells.

We initially carried out two separate dose-response experiments, with one of two different strains, sexes, nitriles, and CYP2E1 inhibitors in each, as an initial evaluation of the general usefulness of the strategy, and a starting point for selecting model parameters. The two strains used were selected for their relevance: the 129S1 strain was developed by Jackson as an adequate control of many genetically modified strains constructed on a 129/SV genetic background, while the Swiss strain is one of the most widely used. We used *cis*-crotononitrile with 129S1 females (Boadas-Vaello et al. 2007) and allylnitrile with Swiss males (Boadas-Vaello et al. 2009), on the basis of the results of our previous studies.

Female 129S1 mice co-treated with DAS showed less acute toxicity and similar vestibular toxicity from *cis*-crotononitrile as mice that were not co-treated. However, the protection was only partial and similar mortality rates were observed at the high *cis*-crotononitrile dose, which indicates a modest rightward shift in the acute toxicity dose-response relationship. Thus, the *cis*-crotononitrile+DAS experiment corroborated the hypothesis that CYP2E1 inhibitors afford protection against nitrile toxicity, but offered a modest improvement in the usefulness of *cis*-crotononitrile as a model for vestibular hair cell ablation in the female 129S1 mouse. In this experiment, only behavioral data were available to demonstrate that DAS causes no vestibular toxicity and does not modify the vestibular toxicity of *cis*-crotononitrile, while both behavioral and

SEM data demonstrated the dose-dependent vestibular toxicity of the nitrile in mice co-treated with DAS. A similar set of data were available for the other dose-response experiment. In this second experiment, TDCE was found to significantly protect male Swiss mice against the acute toxicity of allylnitrile, with only one death and modest body weight effects being observed in the high-dose groups, which had high scores of vestibular dysfunction and extensive to almost complete loss of hair cells. TDCE has a low toxicity, with an acute oral mouse LD₅₀>2,000 mg/kg (Barnes et al. 1985), and no apparent effect was observed with the 3×100 mg/kg dose regime used in the present study. In comparing the nitriles, allylnitrile has a lower LD₅₀ than *cis*-crotononitrile, and the latter apparently results in a more gradual dose-response relationship for vestibular toxicity. However, the lethality data indicate better protection by TDCE against allylnitrile toxicity than DAS against *cis*-crotononitrile toxicity. In addition, allylnitrile is directly available from commercial sources while crotonitrile is only available as a racemic mixture that needs to be distilled (Balbuena and Llorens 2003). For these reasons, we decided to focus on TDCE+allylnitrile for further assessment.

Behavioral and histological data from an initial single-dose experiment using TDCE and 1 mmol/kg of allylnitrile demonstrated the effectiveness of the nitrile in males of the 129S1 strain, the absence of mortality after TDCE co-treatment, the absence of TDCE vestibular toxicity in animals not exposed to allylnitrile, and the absence of any effect of TDCE on the vestibular toxicity of the nitrile. A second single-dose experiment demonstrated the effectiveness of allylnitrile in female 129S1 mice. Again, TDCE caused no vestibular toxicity in animals not exposed to allylnitrile and did not significantly modify the vestibular toxicity of the nitrile. Animals in the previous experiment had not displayed maximal behavioral dysfunction or hair cell loss, and data in male Swiss mice (Fig. 5) suggested that TDCE could protect against the lethal effects of up to 1.5 mmol/kg of allylnitrile; this dose was therefore used in this experiment. However, TDCE offered only marginally significant protection at this high dose of allylnitrile, and an unacceptable level of mortality was recorded in both non-TDCE and TDCE groups of animals.

In the dose-response experiments, intermediate vestibular rating scores (i.e., 5 to 12) were infrequent, as was the observation of moderate loss of utricular hair bundles, as shown in Figure 3D. Intermediate mean values were obtained in association with high variability and reflected mean values from highly affected and largely unaffected animals. Thus, for example, among the male 129S1 mice treated with TDCE+1.0 mmol/kg of allylnitrile, three animals showed bundle counts >65 % of control mean in

association with control-like behavior (rating scores <3), and five showed extensive epithelial damage (<35 % of control mean) in association with high scores (>17) of vestibular dysfunction. The occurrence of partial damage and intermediate vestibular rating scores is nevertheless possible, as observed in a few animals from different treatment groups. From our previous experience with rats (Llorens et al. 1993), mice, and guinea pigs (Soler-Martín et al. 2007), a more gradual dose–response relationship characterizes the vestibular toxicity of IDPN. This is also related with the time lapse between nitrile administration and the appearance of vestibular dysfunction, which is much longer for IDPN than for allylnitrile and *cis*-crotononitrile. These observations suggest that the pharmacokinetic properties of the ototoxic nitrile are key determinants of the steepness of the dose–response curve, which is maximal for allylnitrile. This nitrile will thus show limited suitability for studies requiring loss of a small to moderate percentage of hair cells (e.g., 20 to 60 %), but is much more appropriate for attaining extensive (e.g., 60 to 100 %) hair cell loss. Importantly, the presence of such extensive damage can be reliably predicted by behavioral assessment, and a threshold in behavioral scores (e.g., >14) may be set to select animals with such (>60 %) loss. Considering the data from all four experiments, the individual data show that the 35 animals with scores >14 had bundle counts ≤ 40 % of the corresponding control mean, except for one animal that had a value of 45 %. On a cautionary note, the discriminatory power of the test battery among severely affected animals appeared limited, as similar ratings were obtained for animals with estimated 70 to 100 % loss of hair cells in the utricle.

Previous models for ototoxic cochlear lesioning showed undamaged vestibular epithelia (Taylor et al. 2008; Oesterle et al. 2008). In the present study, we did not assess the effect of *cis*-crotononitrile and allylnitrile on the auditory system. However, in the rat, both nitriles have been demonstrated to cause hearing loss and cochlear hair cell degeneration (Gagnaire et al. 2001; Balbuena and Llorens 2001, 2003), with similar dose–response relationships between cochlear and vestibular damage. Further studies are required to assess whether the present exposure models result in useful cochlear damage in addition to the assessed vestibular damage.

In conclusion, we evaluated a methodological refinement for the use of ototoxic nitriles as tools to cause hair cell ablation in adult mice. Systemic exposure to an ototoxic nitrile, allylnitrile or *cis*-crotononitrile, was assayed with co-administration of pharmacological agents that diminish the acute toxicity of these nitriles without reducing their vestibular toxicity. The protection afforded was limited,

but significant at submaximal doses of the nitriles. These exposure models allowed the study of the relationship between scores in a behavioral test battery for vestibular dysfunction and loss of hair cells as assessed by SEM. The data obtained indicate that high vestibular rating scores in the behavioral test battery predict extensive to complete loss of hair cells in the utricles. This provides a means of selecting animals for subsequent studies of vestibular hair cell regeneration or replacement.

Note

After submission of this manuscript, Golub et al. (2012) have published a transgenic mouse model for targeted ablation of the adult utricle with diphtheria toxin. While the model proposed by Golub et al. has obvious value, the use of nitriles as described in the present paper has the advantage that it does not require adoption and maintenance of a particular mouse strain, so it can be readily adapted to a much wider range of laboratory settings.

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