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ALLYLNITRILE METABOLISM BY CYP2E1 AND OTHER CYPs LEADS TO DISTINCT LETHAL AND VESTIBULOTOXIC EFFECTS IN THE MOUSE[a]

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ABSTRACT. This study addressed the hypothesis that the vestibular or lethal toxicities of allylnitrile depend on CYP2E1-mediated bioactivation. Wild-type (129S1) and CYP2E1-null male mice were exposed to allylnitrile at doses of 0, 0.5, 0.75 or 1.0 mmol/kg (po), following exposure to drinking water with 0 or 1% acetone, which induces CYP2E1 expression. Induction of CYP2E1 activity by acetone in 129S1 mice and lack of activity in null mice was confirmed in liver microsomes. Vestibular toxicity was assessed using a behavioral test battery and illustrated by scanning electron microscopy observation of the sensory epithelia. In parallel groups, concentrations of allylnitrile and cyanide were assessed in blood after exposure to 0.75 mmol/kg of allylnitrile. Following allylnitrile exposure, mortality was lower in CYP2E1-null than in 129S1 mice, and increased after acetone pre-treatment only in 129S1 mice. This increase was associated with higher blood concentrations of cyanide. In contrast, no consistent differences were recorded in vestibular toxicity between 129S1 and CYP2E1-null mice, and between animals pre-treated with acetone or not. Additional experiments evaluated the effect on the toxicity of 1.0 mmol/kg allylnitrile of the non-selective P450 inhibitor, 1-aminobenzotriazole, the CYP2E1-inhibitor, diallylsulfide, and the CYP2A5 inhibitor, methoxsalen. In 129S1 mice, aminobenzotriazole decreased both mortality and vestibular toxicity, whereas diallylsulfide decreased mortality only. In CYP2E1-null mice, aminobenzotriazole and methoxsalen, but not diallylsulfide, blocked allylnitrile-induced vestibular toxicity. We conclude that CYP2E1-mediated metabolism of allylnitrile leads to cyanide release and acute mortality, probably through α-carbon hydroxylation, and hypothesize that epoxidation of the β–γ double bond by CYP2A5 mediates vestibular toxicity.

Keywords: Ototoxicity, Vestibular toxicity, Nitrile, Xenobiotic metabolism, CYP2E1, CYP2A5, Cyanide

INTRODUCTION

Nitriles are common in crop plants and their use in the chemical and pharmaceutical industries is becoming increasingly frequent. Allylnitrile occurs as an industrial product and also as a natural compound that is widely distributed throughout the Cruciferae family (Tanii et al., 2004). The toxic effects of nitriles include acute lethality, osteolathyrism and neurotoxicity (DeVito, 1996). In several animal species, sensory systems have been reported to be major targets for 3,3'-iminodipropionitrile (IDPN), allylnitrile and *cis*-crotononitrile neurotoxicity; toxic effects occur in the vestibular (Llorens et al., 1993; Llorens and Rodríguez-Farré, 1997; Seoane et al., 2001; Balbuena and Llorens 2001, 2003), auditory (Crofton and Knight, 1991; Crofton et al., 1994; Balbuena and Llorens 2001, 2003; Gagnaire et al., 2001), olfactory (Genter et al., 1992; Genter et al., 1994) and visual (Barone et al., 1995; Selye, 1957; Seoane et al., 1999) systems. Recent data demonstrate that these nitriles cause audiovestibular toxicity in many species, including rodents and amphibians (Soler-Martín et al., 2007). IDPN is also known to induce a neurofilamentous proximal axonopathy which particularly affects large myelinated neurons (Chou and Hartmann, 1964; Llorens and Demêmes, 1996). Dimethylaminopropionitrile, which is structurally related to IDPN, has been reported to cause genito-urinary neurotoxicity and polyneuropathy in humans and rats (Pestronk, 2000). Other similar nitriles, namely *trans*-crotononitrile and 2,4-hexadienenitrile, have been shown to cause little or no sensory toxicity, but instead cause selective neuronal degeneration in discrete regions of the rat brain (Seoane et al., 2005; Boadas-Vaello et al., 2005). Despite the interest of the rich variety of neurotoxic effects shown by this group of small nitriles, and the potential relevance to human health, no information is available on their molecular basis.

The data available support the conclusion that cyanide release by nitrile metabolism is a major cause of the acute lethality effect (Willhite and Smith, 1981; Tanii and Hashimoto, 1984), but the role of metabolites in the neurotoxic effects has not been elucidated, in spite of the efforts of several groups (Jacobson et al., 1987, among others). Following initial *in vivo* evidence of P450 involvement in nitrile acute toxicity (Tanii and Hashimoto, 1984; Tanii and Hashimoto, 1986), a major role was hypothesized for the alcohol/acetone-inducible isoform of the P450 cytochrome (CYP2E1) in nitrile metabolism to cyanide (Lewis et al., 1994). The CYP2E1 enzyme has been shown to metabolize a number of small alkyl nitriles (Ghanayem et al., 1999), and is responsible for the initial metabolism of acrylonitrile leading to cyanide release (Wang et al., 2002; Chanas et al., 2003; El Hadri et al., 2005). Furthermore, Genter et al. (1994) provided evidence that metabolism by this CYP isoform may be involved in the olfactory toxicity of IDPN. Recently, we demonstrated that *cis*-crotononitrile is a CYP2E1 substrate in the mouse, but that CYP2E1-mediated metabolism of this nitrile is not necessary for vestibular toxicity; rather, this metabolism constitutes a major pathway for cyanide release and subsequent lethality (Boadas-Vaello et al., 2007). Because *cis*-crotononitrile is α,β-unsaturated, CYP2E1 has been hypothesized to catalyze the epoxidation of the double bond in the molecule, subsequently leading to cyanohydrin formation and cyanide release (Silver et al., 1982; Wang et al., 2002; Ghanayem and Hoffler, 2007). Allylnitrile is the β,γ–unsaturated analog of *cis*-crotononitrile, and shares its vestibular and lethal effects with a higher (2-3 fold) potency. P450-dependent metabolism of allylnitrile to cyanide has been shown *in vitro* (Farooqui et al., 1993), and its toxicity *in vivo* has been shown to be modified by carbon tetrachloride co-administration (Tanii et al., 1993). However, the CYPs involved have yet to be identified, and no role for any metabolites in vestibular toxicity has been

demonstrated. In allylnitrile, cyanide release may result from CYP-mediated α-carbon hydroxylation to form an unstable cyanohydrin (Ohkawa et al., 1972; Silver et al., 1982), while epoxidation of the double bond will not directly lead to cyanide generation (Silver et al., 1982). Either reaction could be mediated by CYP2E1 but also by other CYPs, including CYP2A5 and CYP3a (Fig. 1). CYP2A5 is the mouse ortholog of human CYP2A6, which is known to share with CYP2E1 the ability to catalyze epoxidation of the first double bond in 1,3-butadiene, whereas both CYP2E1 and CYP3A4 mediate epoxidation of the second double bond (Albertini et al., 2003).

The aim of this study was to determine the role of CYP2E1 in the metabolism and toxicity of allylnitrile and to explore the role of other CYPs. To this end, we compared the lethal and vestibular effects of this nitrile on wild-type and CYP2E1 knock-out mice (Lee et al., 1996). Pre-treatment with acetone for CYP2E1 induction was also included in the experimental design. In addition, blood allylnitrile and cyanide concentrations were assessed in order to obtain direct insights into the metabolism of the nitrile in the different experimental groups. Further *in vivo* data were obtained using inhibitors of CYP enzymes. The results demonstrated a major role for CYP2E1 in allylnitrile metabolism leading to cyanide release and lethality, indicating a preferential action of this CYP on the α–carbon of this nitrile. The data also indicated that a different metabolic pathway is involved in the vestibular toxicity of this nitrile, and suggested that this pathway is the epoxidation of the β,γ double bond by CYP2A5.

METHODS

Chemicals and reagents

Allylnitrile (>98%) was purchased from Merck-Schuchard (Hohenbrunn bei München, Germany), and diallylsulfide (97%), 1-aminobenzotriazole (>98%), methoxsalen (8-methoxypsoralen, >98%), p-nitrophenol and NADPH from Sigma-Aldrich (Alcobendas, Spain). Ritonavir was kindly provided by Abbott (Chicago, IL, USA). Other chemicals were of analytical grade as obtained from common commercial sources.

Animals

The care and use of animals were in accordance with Acts 5/1995 and 214/1997 of the Autonomous Community (Generalitat) of Catalonia, and approved by the Ethics Committee on Animal Experiments of the University of Barcelona. CYP2E1-null mice (Lee et al., 1996) were obtained from a local colony established by breeding pairs generously donated by F.J.Gonzalez (US NIH, Bethesda, USA). Because the CYP2E1-null mice were derived from the 129/SV strain, the 129S1/SvImJ mice, with a similar genetic background, were chosen as controls. Therefore, a local colony of this strain was established by breeding pairs obtained from The Jackson Laboratory (Bar Harbor, ME, USA). Sex differences have been found in nitrile metabolism and toxicity in mice (Chanas et al., 2003; Boadas-Vaello et al., 2007); this factor was excluded from the present investigation, in which only males were used. After weaning, mice were housed two to four per cage in standard Macrolon cages (28 x 28 x 15 cm) with wood shavings as bedding and given standard diet pellets (Harlan Teklad Global Diet 2014) *ad libitum*. To obtain tissue and blood samples, mice were anaesthetized with 400 mg/kg chloral hydrate, ip, before killing.

Dosing

Allylnitrile was administered po in 6 ml/kg of corn oil at 0, 0.5, 0.75 or 1.0 mmol/kg. The high dose was selected from pilot studies designed on the basis of literature data (Tanii et al., 1989). The studies included groups of 2-3 mice dosed with 1.0, 1.25 or 1.5 mmol/kg of allylnitrile which were observed for mortality and vestibular dysfunction (data not shown).

To increase the level of CYP2E1 activity, we exposed some groups of mice to acetone, a well known inducer of this cytochrome. Acetone was added at 1% to the drinking water for 1 week before sacrifice or allylnitrile administration (Forkert et al., 1994).

Pharmacological treatments were also used to differentially inhibit different P-450 activities possibly involved in allylnitrile metabolism and toxicity. The non-specific P-450 inhibitor 1-aminobenzotriazole (Mico et al., 1988) was administered ip, in 3 ml/kg of saline, at 50 mg/kg, 2 h before and 24 h after allylnitrile. The selective CYP2E1 inhibitor diallylsulfide (Brady et al., 1991) was administered po, in 6 ml/kg of corn oil, at 200 mg/kg, 24 h and 2 h before, and 24 h after allylnitrile. The CYP2A5 inhibitor methoxsalen (Takeuchi et al., 2003; Visoni et al., 2008) was administered po, suspended in 6 ml/kg of corn oil, at 50 mg/kg, 24 h and 2 h before, and 24 h after allylnitrile. The CYP3A4 inhibitor ritonavir (Bardelmeijer et al., 2002) was administered po, in 4 ml/kg of 25% ethanol-75% propylene glycol, at 12 mg/kg, 30 min before allylnitrile.

Experimental design

The present study includes eight independent experiments. Experiment 1 was designed to corroborate the inducing effect of the acetone treatment on CYP2E1 activity. Four groups of mice were used: wild-type mice with no treatment (129S1 group, n=8), wild-type mice exposed to acetone (129S1+acetone group, n=8), CYP2E1 deficient mice with no treatment (CYP2E1-null group, n=3) and CYP2E1 deficient

mice exposed to acetone (CYP2E1-null+acetone group, n=3). Liver CYP2E1 activity was assessed as described below at one week of acetone exposure.

Experiment 2 determined the role of CYP2E1 in the toxicity of allylnitrile by characterizing the dose-response relationships in both 129S1 and CYP2E1-null strains, pre-treated or not with acetone for CYP2E1 induction. Mice in each of the four strain/pre-treatment conditions (129S1, 129S1+acetone, CY2E1-null and CYP2E1-null+acetone) were administered allylnitrile as detailed in table 1. The animals were assessed for vestibular dysfunction and open field activity on days 0 (pre-test), 2, 7 and 20 after treatment. The experiment was run in five parts, over a 14 month period, with 1-3 animals per dose and condition in each part, due to animal availability and experimenter handling capacity. In order to illustrate the vestibular hair cell loss associated with the vestibular dysfunction observed, selected mice from this study were used for histological analysis at 4-12 weeks after treatment. The four-week period was selected to allow full completion of the hair cell degeneration and epithelial healing processes, while longer times were imposed by experimenter availability. Based on previous studies (Llorens et al., 1993; Llorens and Demêmes, 1994; Soler-Martín et al., 2007; Boadas-Vaello et al., 2008), we were confident that no changes in hair cell density would occur in mice over this period of time, unlike other mammalian species, such as the guinea pig (e.g., Soler-Martin et al., 2007). Fifteen mice were examined, to ensure inclusion of vestibular competent and vestibular deficient animals and of 1-2 animals for each of the four strain/pre-treatment conditions at each dose level of allylnitrile; exceptions were 129S1+acetone animals treated with 1mmol/kg, CYP2E1-null mice treated with 0.5 or 0.75 mmol/kg and the CYP2E1-null+acetone mice treated with 0.50 mmol/kg.

Experiment 3 examined the effects on the blood concentrations of cyanide and

allylnitrile of the changes in CYP2E1 activity. Groups of 129S1, 129S1+acetone, CYP2E1-null and CYP2E1-null+acetone mice (n=10/group) were killed for blood analysis at 20 min after administration of 0.75 mmol/kg of allylnitrile. The 20 min time point was selected because deaths started just after this point in experiment 2.

Experiments 4 and 5 compared the role of CYP2E1 versus other CYPs in allylnitrile toxicity, using 129S1 mice. In experiment 4, 129S1 mice were administered aminobenzotriazole (n=5), 1 mmol/kg allylnitrile (n=8) or both aminobenzotriazole and allylnitrile (n=8). Control vehicles were also given as appropriate. For instance, aminobenzotriazole animals received corn oil (vehicle for allylnitrile) at the same time that animals in the other groups received the allylnitrile solution. In experiment 5, groups of 129S1 mice received diallylsulfide (n=6), 1mmol/kg allylnitrile (n=8), or both diallylsulfide and allylnitrile (n=8). Control vehicles were also administered as appropriate. In both experiments, vestibular dysfunction was assessed on days 0 (pre-test), 2, 7 and 20 after nitrile exposure

Experiment 6 was designed to further ascertain the role of CYP2E1 versus other CYPs in allylnitrile toxicity, using four groups of CYP2E1-null mice. A control group (n=6) was made up of animals treated with either aminobenzotriazole or diallylsulfide (3 animals per inhibitor). The other groups were dosed with 1mmol/kg of allylnitrile (n=6, also receiving saline or oil vehicles of the inhibitors, 3 animals each), aminobenzotriazole and allylnitrile (n=6) and diallylsulfide and allylnitrile (n=6). Vestibular dysfunction was assessed on days 0 (pre-test), 2, 7 and 20 after nitrile exposure.

In experiment 7, we sought support for the hypothesis that CYP3A4 may bioactivate allylnitrile for vestibular toxicity. CYP2E1-null mice were administered ritonavir and 1 mmol/kg allylnitrile (n=5) or ethanol-propylene glycol (vehicle for

ritonavir) and allylnitrile (n=4), and examined for vestibular dysfunction on days 0 (pre-test), 2, 7 and 20 after nitrile exposure.

The last experiment examined the hypothesis that CYP2A5 bioactivates allylnitrile for vestibular toxicity. CYP2E1-null mice were administered methoxsalen (n=10), methoxsalen and 1 mmol/kg allylnitrile (n=9), or allylnitrile alone (n=9). Control vehicles were also administered as appropriate. Vestibular dysfunction was assessed on days 0 (pre-test), 2, 7 and 20 after nitrile exposure.

CYP2E1 activity

CYP2E1 activity was determined by assaying the hydroxylation of *p*-nitrophenol to 4-nitrocatechol in the liver microsomal fraction (Reinke and Moyer, 1985; Wang and Cederbaum, 2006). Briefly, whole livers were disrupted using a teflon-glass homogenizer in 10 ml of 0.15 M KCl. The homogenate was centrifuged for 20 min at 9000 X g, and the supernatant was centrifuged for 30 min at 100 000 X g. The pellet was resuspended in 0.15 M KCl and centrifuged again. The final pellet was resuspended in 4 ml of assay buffer (0.1 M potassium phosphate buffer, pH 6.8, containing 5 mM MgCl2) and stored at – 80 º C. The protein content of the homogenates was determined by the bicinchoninic acid method (Smith et al., 1985), using bovine serum albumin as standard. The CYP2E1 assay was performed by incubating 500 mg of microsomal protein for 30 min at 37ºC in a volume of 1 ml containing 0.1 mM p-nitrophenol, and 1 mM NADPH. The reaction was finished by addition of 0.5 ml of cold 0.6 N HClO4, and the mixture was centrifuged to remove precipitated proteins. One milliliter of the supernatant was added to 0.1ml of 10M NaOH in a spectrophotometer cuvette, and the absorbance read at 546 nm.

Determination of cyanide and allylnitrile in blood

For blood analysis, the thoracic cavity of anaesthetized mice was opened and 600

μl of blood were obtained with a syringe from the left ventricle. Simultaneous analysis of cyanide and allylnitrile was carried out as described in detail elsewhere (Boadas-Vaello et al., 2008). Briefly, the blood was collected in a 6 ml vial containing 400 μL of 0.1 M acetic/acetate buffer (pH= 4.3), which was then stored at -24ºC. These samples were spiked with acetonitrile as internal standard (1.8 μg), acidified with HCl 25%, and analysed by solid-phase microextraction (SPME) gas chromatography coupled to a nitrogen-phosphorus detector (NPD), using a 75 μm carboxen-polydimethylsiloxane fiber (Supelco, Bellefonte, PA, USA) and a J&W (Folsom, CA, USA) Gs-GasPRO column (60 m x 0.32 mm ID). SPME extraction was carried out in the headspace mode at 37ºC for 35 min.

Behavioral analysis

The disturbance of vestibular function was determined using a battery of behavioral tests initially developed for the rat (Llorens et al., 1993; Llorens and Rodríguez-Farré, 1997) and recently adapted to the mouse (Soler-Martín et al., 2007). Briefly, mice were placed for one minute in an open arena (a rat housing cage) and the experimenter rated the animals from 0 to 4 for circling, retropulsion and abnormal head movements. Circling was defined as stereotyped circling ambulation. Retropulsion consisted of backward movement. Head bobbing consisted of intermittent extreme backward extension of the neck. The mice were afterwards rated 0 to 4 by 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 the 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 statistic was obtained by adding up the scores for all behavior patterns.

Open field behavior was assessed as described previously (Soler-Martin et al., 2007). Briefly, locomotor and rearing activities were counted in 5-min sessions in a 50 X 50-cm open field under red light.

Histology

We examined surface preparations of the vestibular sensory epithelia using scanning electron microscopy (SEM), following standard procedures as described elsewhere (Llorens et al., 1993; Seoane et al., 2001; Soler-Martín et al., 2007). 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 epithelia from the inner ear quickly dissected. The samples were then allowed 1.5 h of fixation 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 CO_2. The dried sensory epithelia were coated with carbon and observed in a Hitachi S-4100 Field Emission SEM, or coated with gold and observed in a LEICA 360 SEM.

Statistics

Percentage mortality data were analyzed by contingency tables. Survival over time was analyzed by Kaplan-Meier analysis using the Breslow statistic. Behavioral data were tested with repeated measures MANOVA (Wilks' criterion) with day as the within-subject factor. One-way ANOVA was used for comparison of liver and blood

data. Orthogonal contrasts or Duncan's test were used, when applicable, for post-hoc analysis. In all analyses, the α level was set at 0.05, and SPSS 12.0.1 for Windows was used for statistical processing.

RESULTS

CYP2E1 activity: relationship to strain and acetone pre-treatment.

The CYP2E1 activity recorded in hepatic microsomes is shown in Fig. 2. Significant group differences were obtained ($F(3,18)=132$, $p=0.000$). Pre-treatment with 1 % acetone in the drinking water for 1 week resulted in an approximately 2.5-fold induction of CYP2E1 activity in 129S1 (wild-type) mice, while only residual activity was recorded in CYP2E1-null mice, irrespective of the acetone exposure condition.

Lethal effect of allylnitrile: relationship to strain and acetone pre-treatment.

In the dose-response study, no deaths were recorded in control mice, whereas mortality rates differed in the groups treated with allylnitrile. Mortality was higher in 129S1 than in CYP2E1-null mice, and pre-treatment with 1% acetone increased mortality in the former but not in the latter strain (Fig. 3). Comparison of data from groups exposed to the high dose (1.0 mmol/kg) of allylnitrile indicated that mortality within one day of treatment was significantly higher in the 129S1 mice than in CYP2E1-null mice (chi-square, 1 d.f.= 12, $p=0.001$), and that a significant increase in mortality was associated with acetone pre-treatment within the 129S1 mice (chi-square, 1 d.f.= 6, $p=0.014$). All the 129S1-acetone animals, with the highest CYP2E1 activity, died after 1.0 mmol/kg of allylnitrile. Mortality data after 0.75 mmol/kg of allylnitrile also resulted in significant strain-dependent differences (chi-square, 1 d.f.= 8.3, $p=0.004$), while the effect of acetone pre-treatment on the mortality of the 129S1 animals resulted in a chi-square (1 d.f) of 3.6 ($p=0.058$). CYP2E1-null animals in both acetone pre-treatment conditions were clearly resistant to allylnitrile induced mortality, and only one CYP2E1-null mouse died after the 1.0 mmol/kg dose.

The association of CYP2E1 activity with mortality also included the time course of the effect. In the 129S1+acetone animals, death records occurred after short periods,

starting as early as 20 min and completed by 4 hours, except for one animal dosed with 0.75 mmol/kg, which died after 4 hours but within 24 h. Interestingly, some 129S1+acetone animals presented signs of cyanide intoxication (such as tremor and seizures) 20 min after administration but later fully recovered and did not die or suffer vestibular dysfunction. In the 129S1 animals which had not been pre-treated with acetone, none of the deaths had occurred by 4 hours after treatment and, although most occurred by 24 hours, some of them were recorded on days 2 and 5 after treatment. The only CYP2E1-null animal that died after allylnitrile exposure did so on day 5. Survival analysis of the mice administered high doses of allylnitrile demonstrated significant group differences both at the 0.75 and the 1.0 mmol/kg dose levels (LRS= 12.6 and 20.6, respectively, 3 d.f., both $p<0.01$). Pair-wise comparisons indicated that survival time was shorter in the 129S1+acetone group than in both CYP2E1-null groups after 0.75 mmol/kg (all Breslow statistics>7.0, $p<0.01$). After 1.0 mmol/kg of allylnitrile, 129S1 animals survived less than CYP2E1-null animals (Breslow statistic 3.66, $p=0.05$), while 129S1+acetone animals had a significantly shorter survival than all other groups (all Breslow statistics >5.5, $p<0.05$).

Effects of allylnitrile on behavior scores and vestibular sensory epithelia: relationship with strain and acetone pre-treatment.

In the dose-response study, exposure to allylnitrile caused an increase in vestibular rating scores, indicating loss of vestibular function, in animals from all four strain/pre-treatment conditions (Fig. 4). MANOVA analysis indicated significant day ($F(3, 71)= 45.6$, $p=0.000$) and dose ($F(3, 73)=60.8$, $p=0.000$) main effects, but no effects of strain ($F(1, 73)=0.97$, $p=0.33$) or acetone ($F(1,73)=0.17$, $p=0.68$). Significant interactions were also recorded for strain by dose ($F(3, 73)=5.14$, $p=0.003$), and for day by strain ($F(3,71)=6.4$, $p=0.001$) and day by dose ($F(9,173)=13.9$, $p=0.000$. Although the overall

appearance of the behavioral data (Fig. 4A), and one statistically significant difference between groups (Fig. 4B, D) indicated that CYP2E1-null mice had a greater loss of vestibular function than 129S1 mice after 1.0 mmol/kg of allylnitrile, it should be noted that only 2 mice treated with this dose were available for analysis in the 129S1 group and none in the 129S1+acetone group. Nor were group differences observed at the dose level of 0.75 mmol/kg (Fig. 4C). Thus, the loss of vestibular function in CYP2E1-null animals demonstrated that this cytochrome is not necessary for the vestibular toxicity of allylnitrile, but any further conclusion regarding the involvement of this enzyme in this effect was weakened by the mortality occurring in the experiment.

Vestibular toxicity has been associated with increased locomotor activity and decreased rearing activity in both rats (Llorens et al., 1993; Llorens and Rodríguez-Farré, 1997) and CD-1 mice (Soler-Martín et al., 2007). In the present experiment, the 129S1 animals showed a basal rearing activity of only 1.8$\pm$0.4 rears/5-min (X$\pm$SEM, n=56). In addition, strain differences were found in basal locomotor activity (76$\pm$7 for the 129S1 vs. 213$\pm$6 for the CYP2E1-null, counts/5-min; n=56 and 52, respectively), and no consistent hyperactivity was observed in the mice with profound vestibular loss, which were found almost exclusively in the CYP2E1-null and CYP2E1-null+acetone groups treated with 1.0 mmol/kg of allylnitrile (see Fig. 4). The open field data were not used further to compare the vestibular toxicity of allylnitrile in the different groups of mice.

The mice examined to illustrate the hair cell loss associated with the vestibular dysfunction described above had received vestibular rating scores on day 21 of 0-1 (n=9), 5 (n=1) and 16-22 (n=5). Control mice and mice treated with allylnitrile but with low vestibular rating scores of 0-1 showed a high density of hair cell bundles in the vestibular sensory epithelia (Fig. 5A), as previously described (Soler-Martin et al.,

2007). In contrast, animals with high vestibular rating scores displayed markedly altered vestibular epithelia, with a complete or almost complete loss of hair cell bundles in their cristas (Fig. 5B,C), and extensive loss of bundles and abnormal configuration in most of the bundles remaining in their utricles and saccules. The association of high vestibular rating scores and vestibular pathology after allylnitrile exposure was observed in animals in both 129S1 (Fig. 5B) and CYP2E1-null strains (Fig. 5C), as well as with (Fig. 5C) and without (Fig. 5B) acetone pre-treatment. In no case was there any evidence of hair cell regeneration in the form of short hair bundles.

Blood concentrations of allylnitrile and cyanide: relationship to strain and acetone pre-treatment.

The effects of strain and acetone pre-treatment on the concentrations of cyanide and allylnitrile in blood at 20 min are shown in Fig. 6. At this time point, mice from the 129S1+acetone group showed signs of toxicity compatible with cyanide intoxication, such as tremor and convulsions; deaths had been recorded beyond this time point in the previous experiment. Significant group differences were found for both allylnitrile ($F(3, 36)=7.68$, $p=0.000$) and cyanide ($F(3, 36)=9.45$, $p=0.000$). Blood concentrations of allylnitrile were significantly lower in both 129S1 groups than in both CYP2E1-null groups (Fig. 6A). Cyanide concentrations (Fig. 6B) were significantly higher in the 129S1+acetone group than in the 129S1, the CYP2E1-null and the CYP2E1-null+acetone groups.

Effects of 1-aminobenzotriazole and diallylsulfide on allylnitrile toxicity.

Co-treatment of 129S1 mice with the non-specific P-450 inhibitor 1-aminobenzotriazole or the selective CYP2E1 inhibitor diallylsulfide had differing effects on allylnitrile lethal and vestibular toxicities (Fig. 7). Aminobenzotriazole increased survival in mice exposed to 1.0 mmol/kg of allylnitrile, reducing the 50%

mortality recorded at 24 h in animals exposed to the nitrile alone to zero up to day 5 in co-treated animals (Fig. 7A). Aminobenzotriazole also caused a marked inhibition of the vestibular toxicity of allylnitrile (Fig. 7B). MANOVA analysis of the behavioral data indicated significant day ($F(3, 9)= 8.38$, $p=0.006$), treatment ($F(2, 11)=8.10$, $p=0.007$) and day by treatment ($F(6, 18)=3.14$, $p=0.028$) effects. Significant group differences were recorded on all days after treatment (all $F(2,13)>7.9$, $p<0.008$). Reduced mortality was also observed in animals given allylnitrile with diallylsulfide co-treatment (Fig. 7A) whereas the vestibular toxicity was slightly enhanced rather than reduced by this inhibitor (Fig. 7C). MANOVA analysis of the behavioral data indicated significant day ($F(3, 10)= 1437$, $p=0.000$), treatment ($F(2, 12)=143$, $p=0.000$) and day by treatment effects ($F(6, 20)=60.62$, $p=0.000$). Significant group differences were recorded on all days after treatment (all $F(2,14)>44$, $p=0.000$).

In CYP2E1-null mice, allylnitrile exposure (1.0 mmol/kg) caused no mortality, but a significant loss of vestibular function which was prevented by co-treatment with aminobenzotriazole, but not by co-treatment by diallylsulfide (Fig. 8). MANOVA analysis indicated significant day ($F(3, 18)= 362$, $p=0.000$), treatment ($F(3, 20)=256$, $p=0.000$) and day by treatment ($F(9, 44)=24$, $p=0.000$) effects. Significant group differences were recorded on all days after treatment (all $F(3,20)>58$, $p<0.000$).

Taken together with the results of the previous experiments, these results demonstrated that at least one process inhibited by aminobenzotriazole, probably a cytochrome P-450 activity, is involved in allylnitrile vestibular toxicity, but that CYP2E1 activity is not involved.

Effects of methoxsalen and ritonavir on allylnitrile toxicity.

On theoretical grounds (see Introduction), we hypothesized that CYP3A4 or CYP2A5 enzymes could be responsible for allylnitrile bioactivation for vestibular

toxicity; if so, co-administration of inhibitors of these CYPs with allylnitrile should decrease this effect. We chose CYP2E1-null animals to perform these experiments, thus avoiding high mortality rates even when using a high allylnitrile dose (1 mmol/kg). Co-administration of the CYP3A4 inhibitor, ritonavir, did not result in a significant decrease in the vestibular toxicity (data not shown). However, the vestibular toxicity of allylnitrile was significantly inhibited by the CYP2A5 inhibitor methoxsalen (Fig. 9). MANOVA analysis indicated significant day ($F(3, 23)= 25.7$, $p=0.000$), treatment ($F(2, 25)= 44.8$, $p=0.000$) and day by treatment ($F(6, 46)= 10.1$, $p=0.000$) effects. Significant group differences were recorded on all days after treatment (all $F(2,25)>35$, $p<0.000$).

DISCUSSION

The present study first tested the hypothesis that metabolism of allylnitrile by CYP2E1 is a bioactivation step for the lethal and/or the vestibular effects of this compound. By comparing the effects of allylnitrile in untreated wild type animals, in animals with an increased CYP2E1 activity, and in animals lacking the CYP2E1 enzyme, we demonstrated that this cytochrome-P450 isoform is not necessary for vestibular toxicity but is involved in allylnitrile-induced lethality. Once the role of CYP2E1 was established, we hypothesized that other CYPs such as CYP2A5 or CYP3A4 could be involved in the vestibular effect. Then, we collected pharmacological data supporting the hypotheses that CYP activity is indeed necessary for the vestibular toxicity of allylnitrile, and that CYP2A5 plays a bioactivating role in this effect.

Wild type and CYP2E1-null animals were maintained as two separate colonies, owing to operational restraints. Although both strains derive from a common 129S origin (Lee et al., 1996), and are thus genetically similar, the existence of strain differences not related to the presence or absence of the *cyp2e1* gene cannot be ruled out. In the study we therefore included 129S1 animals pre-treated with acetone in order to clearly identify the strain differences due to CYP2E1 activity. CYP2E1 induction by acetone is a well established and quite specific response (e.g., Forkert et al., 1994), and was consistently demonstrated in liver microsomes. The CYP2E1-null+acetone groups of animals allowed us to control for acetone effects not related to CYP2E1 activity.

The enhancement of the lethal effects of allylnitrile by acetone pretreatment in 129S1 animals only, and the reduced mortality in CYP2E1-null mice, demonstrated a bioactivating role for the CYP2E1 enzyme in this effect. By the time at which deaths were initially recorded in the 129S1+acetone animals, blood cyanide concentrations in this group were in the range (low hundreds of μM) associated with mortality after acute

acrylonitrile exposure in rats (Benz and Nerland, 2005), *cis*-crotononitrile exposure in mice (Boadas-Vaello et al., 2007) and cyanide exposure in humans (Baud et al., 1996). We therefore conclude that allylnitrile is metabolized by CYP2E1 *in vivo*, and that this metabolic action leads to cyanide release and acute lethality, as previously demonstrated for acrylonitrile (Benz and Nerland, 2005; Wang et al., 2002) and *cis*-crotononitrile (Boadas-Vaello et al., 2007). In addition to this role of CYP2E1, other enzymes must be able to lead the metabolism of allylnitrile to cyanide, because mean blood concentrations of cyanide in the CYP2E1-null mice were as high as 25-40% of those in 129S1+acetone animals. We did not attempt to identify these alternative cyanogenic pathways. The short time taken to reach lethal cyanide concentrations after allylnitrile administration is not surprising, as it corroborates the findings in rats given acrylonitrile, in which a first wave of toxic effects was shown to be caused by cyanide (Benz and Nerland, 2005). Because CYP2E1 plays an essential role in cyanide release from acrylonitrile (Wang et al., 2002), and as this enzyme is expressed in the gastrointestinal tract and the liver (sites where it is efficiently induced by ethanol or acetone, Shimizu et al., 1990; Forkert et al., 1994), it seems likely that both acrylonitrile and allylnitrile are quickly metabolized to cyanide upon absorption in wild-type animals. Also relevant to this issue is the observation of 129S1+acetone animals with signs of cyanide intoxication soon after allylnitrile administration, but which fully recovered afterwards.

The conclusion that allylnitrile is metabolized by CYP2E1 to cause cyanide release and mortality is in agreement with previous reports for *cis*-crotononitrile (Boadas-Vaello et al., 2007). However, there is a major difference between these two nitriles. In the case of *cis*-crotononitrile, it has been hypothesized that CYP2E1 catalyzes the epoxidation of the α,β-double bond in the molecule, subsequently leading

to cyanohydrin formation and cyanide release (Silver et al., 1982; Wang et al., 2002). In contrast, two actions of CYP2E1 were possible on the β,γ–unsaturated allylnitrile (Fig 1): either α-carbon hydroxylation (Ohkawa et al., 1972; Silver et al., 1982) or epoxidation of the double bond (Silver et al., 1982). Because only the first of these two actions would form an unstable cyanohydrin and thus be cyanogenic, the present results clearly indicate that this is the main action of this CYP on this nitrile.

In the dose-response study, the role of CYP2E1 in the acute lethality of allylnitrile was conclusively established, but whether or not it plays a partial role in the vestibular toxicity was not clear. However, the data showed vestibular toxicity for allylnitrile in CYP2E1-null mice, demonstrating that CYP2E1-activity is not required for this toxic effect. The excessive mortality in the wild-type groups ruled out any firm conclusion on the issue of possible dose-response differences between the CYP2E1-null, 129S1, and 129S1+ acetone groups of mice for vestibular toxicity. Therefore, in order to elucidate whether allylnitrile required CYP-mediated metabolism for vestibular toxicity and whether the CYP2E1 was at least partially involved in this bioactivation, we compared the modulation of the lethal and vestibular effects by aminobenzotriazole, a non-specific P450 inhibitor, and diallylsulfide, a selective CYP2E1 inhibitor, in both 129S1 and CYP2E1-null mice. Although the use of pharmacological inhibitors may sometimes lead to difficulties of interpretation, combining genetic and pharmacological manipulations offered a more robust approach. Thus, the differential effects of these inhibitors in the toxicities of allylnitrile in both strains strongly support the hypotheses that CYP-mediated metabolism is required for the vestibular toxicity, and that CYP2E1 does not play a role in this bioactivation.

If a CYP other than 2E1 mediates allylnitrile vestibular toxicity, one possibility is that this CYP catalyzes the α-carbon hydroxylation and subsequent cyanide release

locally in the inner ear. However, this hypothesis requires that the same metabolites generated elsewhere in the body by CYP2E1 completely fail to add to this effect. A simpler hypothesis would be that the vestibulotoxic pathway differs from the cyanogenic pathway, with the epoxidation of the double bond being a major candidate (Fig. 1). Hypothesizing that either CYP3A4 or CYP2A5 could mediate this epoxidation to bioactivate allylnitrile for vestibular toxicity, we obtained support for a role of this kind for CYP2A5 by demonstrating inhibition of the vestibular effect by the CYP2A5 inhibitor methoxsalen. Additional work is needed to demonstrate whether epoxidation of the double bound is necessary for the vestibular toxicity of allylnitrile, and whether CYP2A5 is the main CYP involved in this pathway. Still unsolved is the nature of the ultimate toxic compound: the epoxide would be a good candidate, but it could be further metabolized by epoxide hydrolases to the corresponding diol or conjugate with glutathione (Silver et al., 1982). One attractive possibility is that the vestibular toxicity may be dependent on the presence of a hydroxyl group at the β-carbon, because it has been postulated that a similar structure is formed during IDPN metabolism (Jacobson et al., 1987).

In summary, a number of nitriles are known to cause vestibular toxicity, but the identity of the ultimate toxic molecule(s) remains to be identified. The results presented here provide support for the hypothesis that different metabolic pathways mediate the lethal and vestibular effects of allylnitrile. The data demonstrate that allylnitrile is a CYP2E1 substrate, and that this action mediates cyanide release and acute lethality but not vestibular toxicity; this cyanogenic role of CYP2E1 indicates a preferential action through α-carbon hydroxylation rather than through epoxidation of the β–γ double bond. In addition, data in this study support the hypothesis that other CYP-mediated

pathways are required for the vestibular effect of allylnitrile, and that these probably include CYP2A5-mediated epoxidation of its double bond.

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LEGENDS FOR FIGURES:

Figure 1. Hypothesized pathways for the CYP-mediated oxidative metabolism of allylnitrile. Hydroxylation at the alpha carbon would generate an unstable cyanohydrin, subsequently decomposing into 2-propenal (acrolein) and cyanide. Epoxidation of the beta-gamma double bound would not be directly cyanogenic.

Figure 2. Effects of acetone pre-treatment on liver microsomal CYP2E1 activity in 129S1 and CYP2E1-null mice. CYP2E1 activity was determined by measuring the rate of *p*-nitrophenol hydroxylation to 4-nitrochatecol by liver microsomes after one week of exposure to 0 or 1% acetone in the drinking water. a, b, c: groups not sharing a letter differed significantly ($p<0.05$, Duncan's tests).

Figure 3. Cumulative lethality induced by allylnitrile in male mice of the 129S1 and CYP2E1-null strains with or without pre-treatment with 1% acetone in drinking water for one week before nitrile administration. Inducing the CYP2E1 activity with acetone resulted in increased mortality in 129S1 mice, while lack of CYP2E1 activity in both CYP2E1-null and CYP2E1-null+acetone groups was associated with reduced mortality in comparison to 129S1 mice.

Figure 4. Effects of allylnitrile (0.5, 0.75 and 1.0 mmol/kg) on the vestibular function test in 129S1 and CYP2E1-null male mice with or without pre-treatment with 1% acetone in drinking water for one week before nitrile administration. **A:** Complete set of mean group vestibular rating scores. The maximum score is 24. Parts of the data in A are shown again in panels B, C, and D, including standard error bars and statistical results. **B:** Dose-response relationship of allylnitrile effects in the four experimental groups at day 2 after exposure. **C:** Time-course of the effects of 0.75 mmol/kg of allylnitrile. **D:** Time-course of the effects of 1.0 mmol/kg of

allylnitrile. Numbers within the graphs are surviving mice at the corresponding dose, group, and time. Note that the data from the 129S1 animals exposed to 1.0 mmol/kg correspond to only two survivors, while there are no data from 129S1+acetone animals treated with this dose, because all the animals in this group died within 4 h of treatment. a,b: groups not sharing a letter are significantly different, $p < 0.05$, Duncan's test.

Figure 5. Effects of allylnitrile on the vestibular sensory epithelia of the mouse, as observed by SEM at 4-12 weeks after exposure. (A) Control crista. Note the dense distribution of hair bundles over the surface of the sensory epithelium (arrow). (B) Crista of a 129S1 mouse treated with 0.75 mmol/kg of allylnitrile displaying a major loss of vestibular function (vestibular rating score at day 21 was 16), and killed at day 34 after dosing. Note the very few hair cell bundles remaining in the epithelium (arrow). A slightly higher density of hair bundles remained in the utricles and saccules from this animal. (C) Crista of a CYP2E1-null+acetone mouse dosed with 1.0 mmol/kg of allylnitrile and killed at day 37 after dosing; this animal suffered the deepest vestibular dysfunction (vestibular rating score at day 21 was 22). No hair cell bundles remain in the epithelium. The very few hair bundles remaining in the utricles from this animal showed abnormal morphologies. Scale bars: 50 μm.

Figure 6. Concentrations of allylnitrile (A) and cyanide (B) in blood of mice at 20 min after oral exposure to 0.75 mmol/kg of allylnitrile in 129S1 and CYP2E1-null mice which had been pre-treated or not with 1% of acetone in drinking water to induce CYP2E1 activity. a,b: groups not sharing a letter differed significantly ($p<0.05$, Duncan's tests).

Figure 7. Effects of the non-selective P450 inhibitor 1-aminobenzotriazole (ABT) and the CYP2E1-selective inhibitor diallylsulfide (DAS) on the lethal and vestibular toxicities of allylnitrile (1.0 mmol/kg) in male 129S1 mice. (A) Cumulative lethality induced by allylnitrile (two groups, co-administered the different vehicles used for the inhibitors), ABT, DAS, ABT+allylnitrile and DAS+allylnitrile. Both inhibitors reduced allylnitrile-induced mortality. (B) Reduced vestibular toxicity of allylnitrile by co-treatment with 1-aminobenzotriazole. *: significantly different from the other two groups ($p<0.05$, Duncan's tests). (C) Lack of protective effect of diallylsulfide against allylnitrile-induced vestibular toxicity. a,b,c: groups not sharing a letter differed significantly ($p<0.05$, Duncan's tests).

Figure 8. Effects of the non selective P450 inhibitor 1-aminobenzotriazole (ABT) and the CYP2E1-selective inhibitor diallylsulfide (DAS) on the vestibular toxicity of allylnitrile (1.0 mmol/kg) in male CYP2E1-null mice. Aminobenzotriazole, but not diallylsulfide, blocked the vestibular toxicity. a,b,c: groups not sharing a letter differed significantly ($p<0.05$, Duncan's tests).

Figure 9. Effect of the CYP2A5 inhibitor methoxsalen on the vestibular toxicity of allylnitrile (1.0 mmol/kg) in male CYP2E1-null mice. Methoxsalen blocked the vestibular toxicity. a,b: groups not sharing a letter differed significantly ($p<0.05$, Duncan's tests).

TABLE 1. Numbers of mice used to compare the dose-response curve of the lethal and vestibular effects of allylnitrile in animals differing in CYP2E1 activity.

Experimental groups	**Allylnitrile dose (mmol/kg)**			
	0.0	**0.5**	**0.75**	**1.0**
129S1	6	7	9	6
129S1-Acetone	6	7	9	6
CYP2E1-null	6	7	7	6
CYP2E1-null+Acetone	6	7	7	6

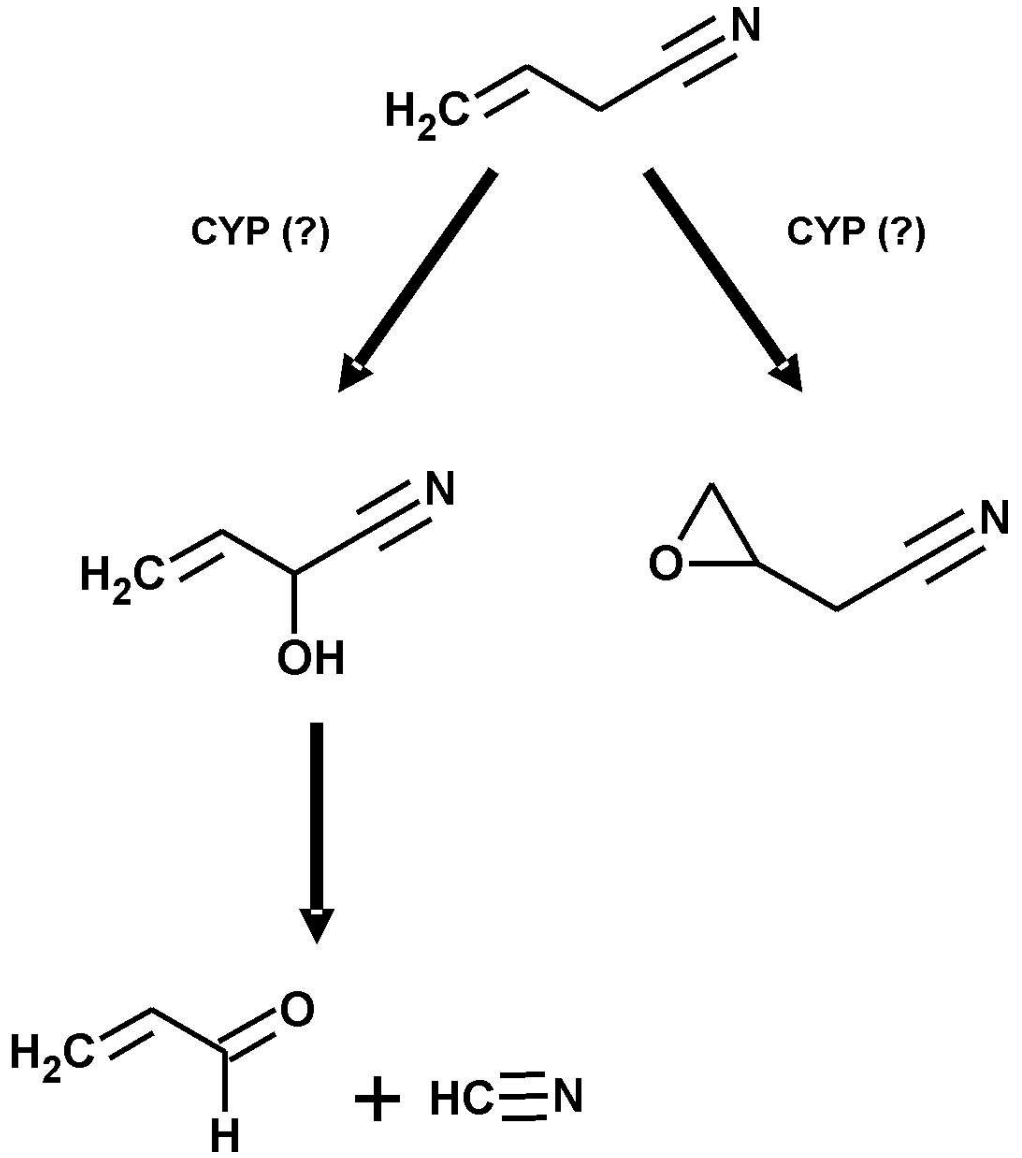

Figure 1. Hypothesized pathways for the CYP-mediated oxidative metabolism of allylnitrile. Hydroxylation at the alpha carbon would generate an unstable cyanohydrin, subsequently decomposing into 2-propenal (acrolein) and cyanide. Epoxidation of the beta-gamma double bound would not be directly cyanogenic.

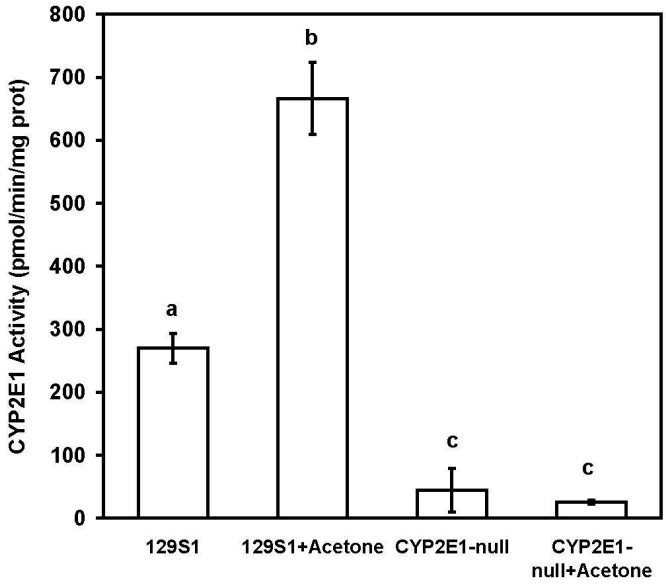

Figure 2. Effects of acetone pre-treatment on liver microsomal CYP2E1 activity in 129S1 and CYP2E1-null mice. CYP2E1 activity was determined by measuring the rate of p-nitrophenol hydroxylation to 4-nitrochatecol by liver microsomes after one week of exposure to 0 or 1 % acetone in the drinking water. a, b, c: groups not sharing a letter differed significantly ($p<0.05$, Duncan's tests).
128x79mm (300 x 300 DPI)

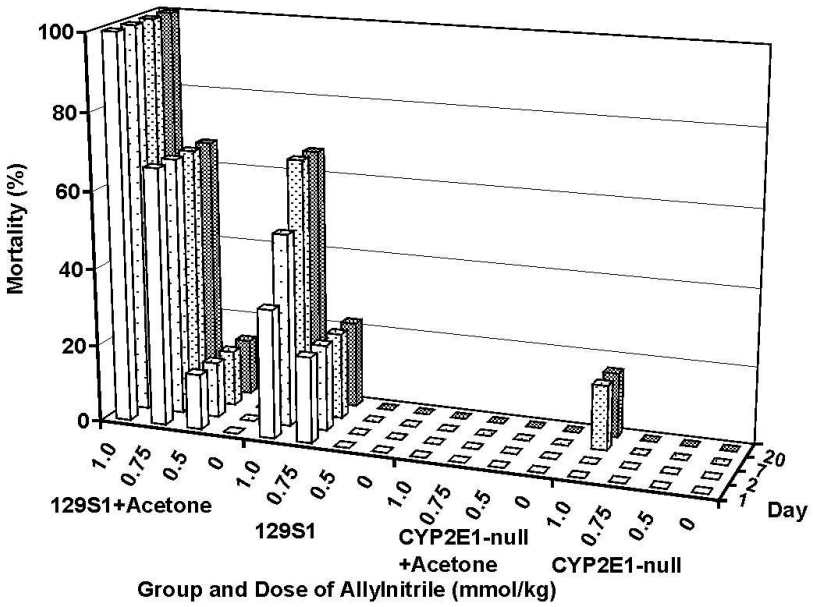

Figure 3. Cumulative lethality induced by allylnitrile in male mice of the 129S1 and CYP2E1-null strains with or without pre-treatment with 1% acetone in drinking water for one week before nitrile administration. Inducing the CYP2E1 activity with acetone resulted in an increased mortality in 129S1 mice, while lack of CYP2E1 activity in both CYP2E1-null and CYP2E1-null+acetone groups was associated with reduced mortality in comparison to 129S1 mice.

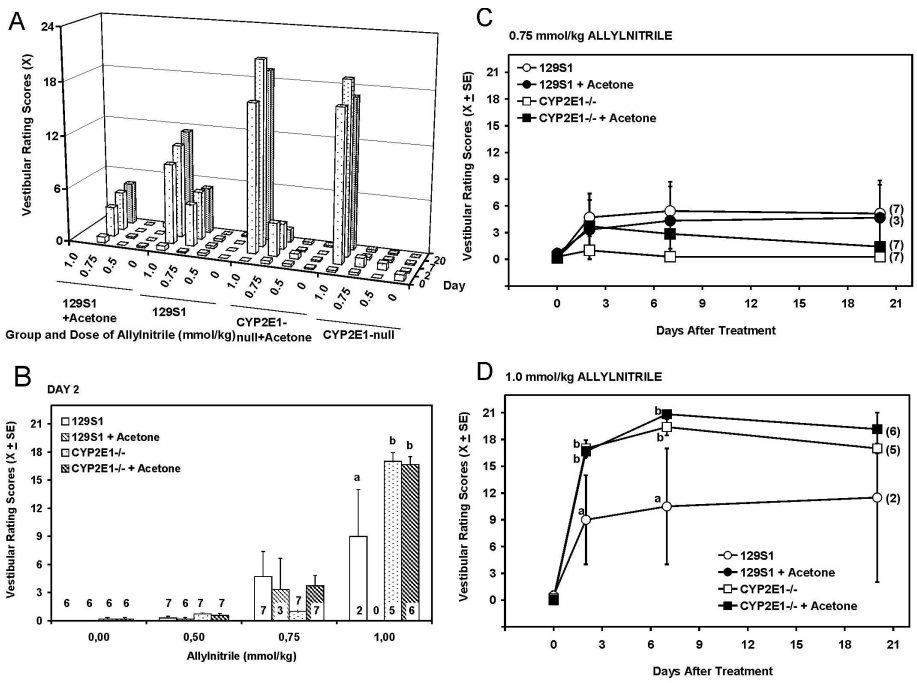

Figure 4. Effects of allylnitrile (0.5, 0.75 and 1.0 mmol/kg) on the vestibular function test in 129S1 and CYP2E1-null male mice with or without pre-treatment with 1% acetone in drinking water for one week before nitrile administration. A: Complete set of mean group vestibular rating scores. The maximum score is 24. Parts of the data in A are shown again in panels B, C, and D, including standard error bars and statistical results. B: Dose-response relationship of allylnitrile effects in the four experimental groups at day 2 after exposure. C: Time-course of the effects of 0.75 mmol/kg of allylnitrile. D: Time-course of the effects of 1.0 mmol/kg of allylnitrile. Numbers within the graphs are surviving mice at the corresponding dose, group, and time. Note that the data from the 129S1 animals exposed to 1.0 mmol/kg correspond to only two survivors, while there are no data from 129S1+acetone animals treated with this dose, because all the animals in this group died within 4 h of treatment. a,b: groups not sharing a letter are significantly different, $p < 0.05$, Duncan's test.

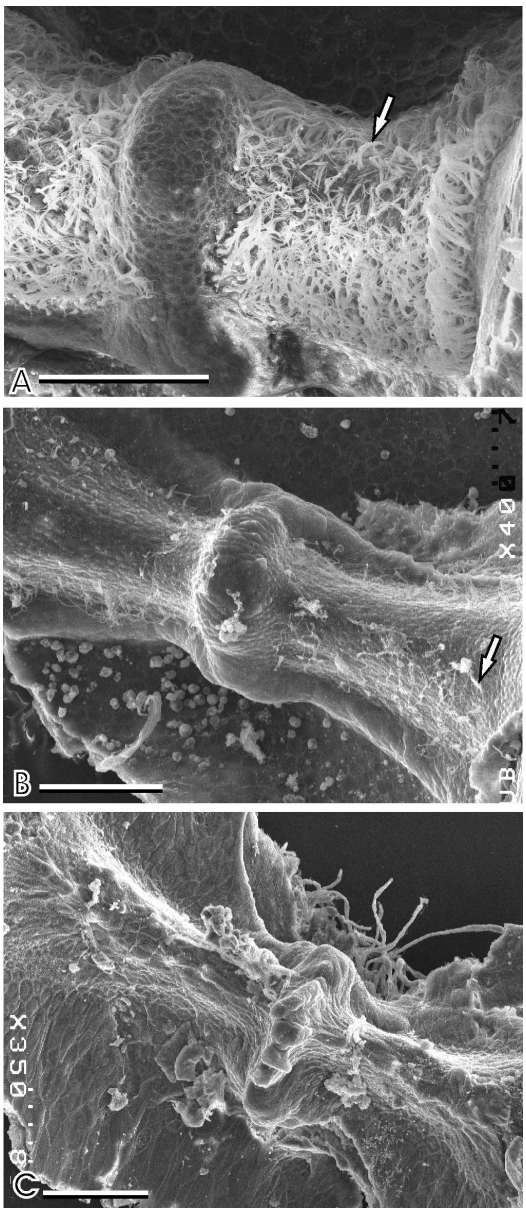

Figure 5. Effects of allylnitrile on the vestibular sensory epithelia of the mouse, as observed by SEM at 4-12 weeks after exposure. (A) Control crista. Note the dense distribution of hair bundles over the surface of the sensory epithelium (arrow). (B) Crista of a 129S1 mouse treated with 0.75 mmol/kg of allylnitrile displaying a major loss of vestibular function (vestibular rating score at day 21 was 16), and killed at day 34 after dosing. Note the very few hair cell bundles remaining in the epithelium (arrow). A slightly higher density of hair bundles remained in the utricles and saccules from this animal. (C) Crista of a CYP2E1-null+acetone mouse dosed with 1.0 mmol/kg of allylnitrile and killed at day 37 after dosing; this animal suffered the deepest vestibular dysfunction (vestibular rating score at day 21 was 22). No hair cell bundles remain in the epithelium. The very few hair bundles remaining in the utricles from this animal showed abnormal morphologies. Scale bars: 50 μm.
85x197mm (300 x 300 DPI)

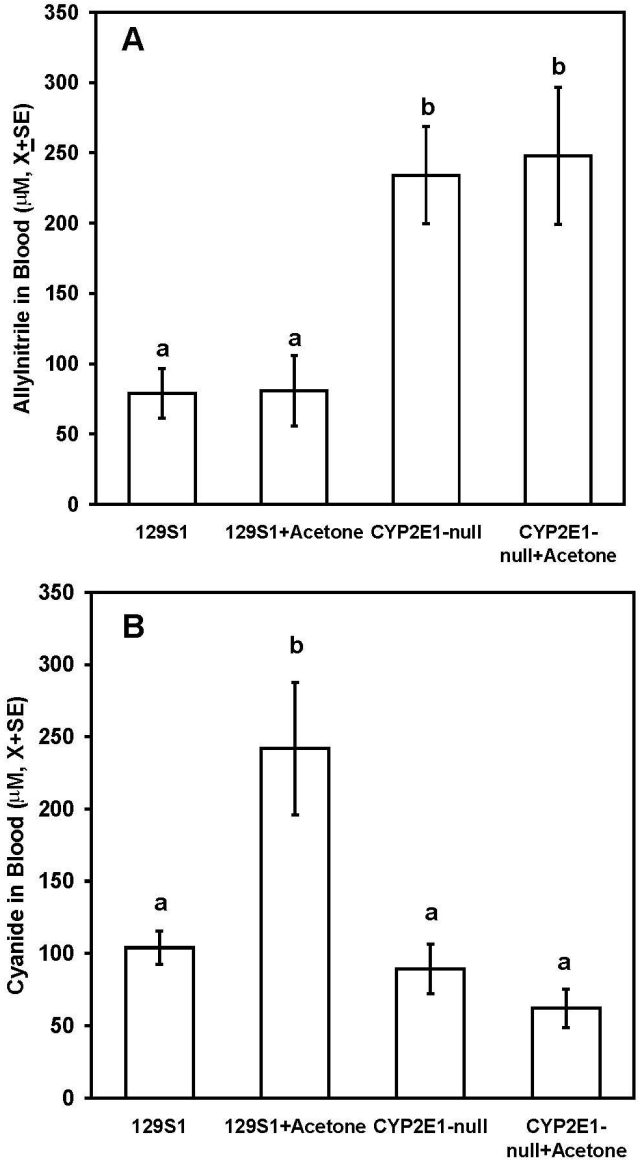

Figure 6. Concentrations of allylnitrile (A) and cyanide (B) in blood of mice at 20 min after oral exposure to 0.75 mmol/kg of allylnitrile in 129S1 and CYP2E1-null mice which had been pre-treated or not with 1% of acetone in drinking water to induce CYP2E1 activity. a,b: groups not sharing a letter differed significantly ($p<0.05$, Duncan's tests).
129x155mm (300 x 300 DPI)

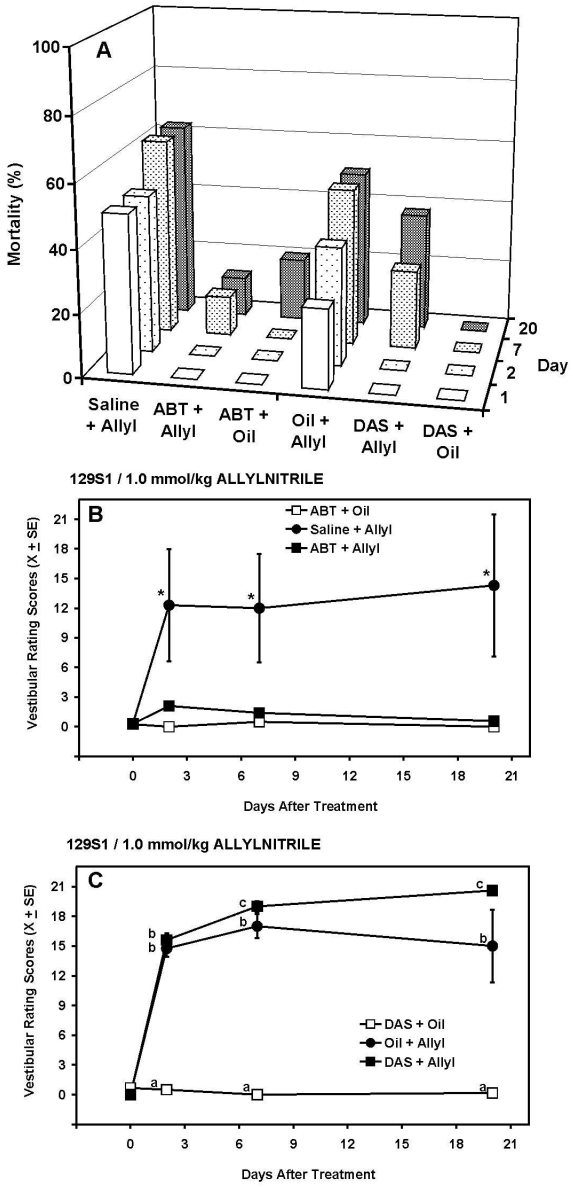

Figure 7. Effects of the non-selective P450 inhibitor 1-aminobenzotriazole (ABT) and the CYP2E1-selective inhibitor diallylsulfide (DAS) on the lethal and vestibular toxicities of allylnitrile (1.0 mmol/kg) in male 129S1 mice. (A) Cumulative lethality induced by allylnitrile (two groups, co-administered the different vehicles used for the inhibitors), ABT, DAS, ABT+allylnitrile and DAS+allylnitrile. Both inhibitors reduced allylnitrile-induced mortality. (B) Reduced vestibular toxicity of allylnitrile by co-treatment with 1-aminobenzotriazole. *: significantly different from the other two groups ($p<0.05$, Duncan's tests). (C) Lack of protective effect of diallylsulfide against allylnitrile-induced vestibular toxicity. a,b,c: groups not sharing a letter differed significantly ($p<0.05$, Duncan's tests).
124x190mm (300 x 300 DPI)

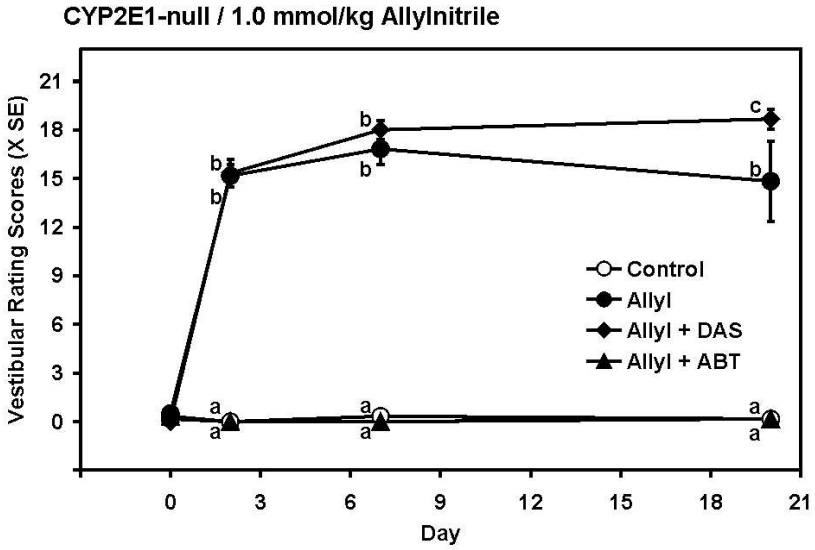

Figure 8. Effects of the non selective P450 inhibitor 1-aminobenzotriazole (ABT) and the CYP2E1-selective inhibitor diallylsulfide (DAS) on the vestibular toxicity of allylnitrile (1.0 mmol/kg) in male CYP2E1-null mice. Aminobenzotriazole, but not diallylsulfide, blocked the vestibular toxicity. a,b,c: groups not sharing a letter differed significantly ($p<0.05$, Duncan's tests).
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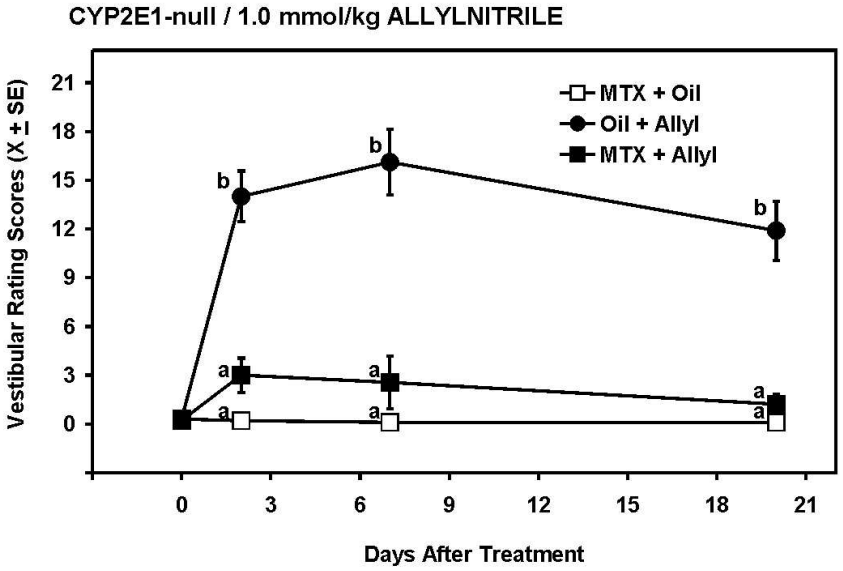

Figure 9. Effect of the CYP2A5 inhibitor methoxsalen on the vestibular toxicity of allylnitrile (1.0 mmol/kg) in male CYP2E1-null mice. Methoxsalen blocked the vestibular toxicity. a,b: groups not sharing a letter differed significantly ($p<0.05$, Duncan's tests).
104x65mm (300 x 300 DPI)