Chimeric TK-NOG Mice: A Predictive Model for Cholestatic Human Liver Toxicity

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Abstract

Due to the substantial inter-species differences in drug metabolism and drug disposition, drug-induced liver injury (DILI) in humans is often not predicted by studies performed in animal species. For example, a drug (bosentan) used to treat pulmonary artery hypertension caused unexpected cholestatic liver toxicity in humans, which was not predicted by pre-clinical toxicology studies in multiple animal species. Here, we demonstrate that TK-NOG mice with humanized livers have a humanized profile of biliary excretion of a test (cefmetazole) drug, which was shown by an in situ perfusion study to result from inter-species differences in the rate of biliary transport and in liver retention of this drug. We also found that readily detectable cholestatic liver injury develops in TK-NOG mice with humanized livers after one week of treatment with bosentan (160, 32 or 6 mg/kg/day PO), while liver toxicity did not develop in control mice after one month of treatment. The laboratory and histologic features of bosentan-induced liver toxicity in humanized mice mirrored that of human subjects. Since DILI has become a significant public health problem, drug safety could be improved if pre-clinical toxicology studies were performed using humanized TK-NOG.
Introduction

Drug-induced liver injury (DILI) has become a leading cause of acute liver failure in several western countries, and is the most common reason for regulatory actions after drug approval (Ostapowicz et al. 2002; Watkins and Seeff 2006). Inter-species differences in the drug metabolism and disposition pathways used by humans and animal species [reviewed in (Peltz 2013) (Williams et al. 2008)] have limited the predictive utility of animal toxicology studies. The results obtained from in vitro systems and from in vivo animal testing have not always accurately predicted the drug metabolism (Anderson et al. 2009; Leclercq et al. 2009; Walker et al. 2009) or transporter-mediated drug clearance (Williams et al. 2008) pathways in humans. Because of this, drugs that produced minimal toxicity in animal studies have sometimes caused significant DILI in humans. The fatalities occurring in 7 of 15 human subjects that were treated with fialuridine provides a striking example of an unexpected DILI that was not predicted by toxicology studies in animal species (Manning and Swartz 1995; McKenzie et al. 1995).

Although its toxicity was less severe, bosentan, which is an endothelin receptor antagonist used for the treatment of pulmonary arterial hypertension (Rubin et al. 2002), provides another example of un-anticipated DILI in humans (Fattinger et al. 2001). Bosentan did not cause liver toxicity in pre-clinical animal models, but it caused dose-dependent and reversible liver damage in ~10% of treated humans, which is manifested by elevated transaminase levels (Fattinger et al. 2001; Humbert et al. 2007). This has significantly limited its therapeutic utility, and patients taking bosentan must undergo monthly liver function monitoring. Bosentan-mediated inhibition of bile salt export pump (BSEP) activity interferes with bile acid secretion (Fattinger et al. 2001), which is thought to be responsible for its cholestatic toxicity. However, since bosentan inhibits both rodent and human BSEP (Fouassier et al. 2002), the species-specific difference in susceptibility to bosentan-induced liver toxicity can not be explained by BSEP inhibition alone.

More broadly, species-specific differences in drug transport make it difficult to accurately assess a drug’s potential for causing cholestatic hepatotoxicity in humans. Pharmaceutical companies are now producing drugs with high aqueous solubility; which further compounds the problem, since their elimination is more dependent on transporter-mediated biliary excretion pathways (Luo et al. 2010).

Chimeric mice with ‘humanized livers’ were produced to generate a more predictive platform, which would improve drug safety. The humanized liver is produced by transplantation of human liver cells into mice with genetically-engineered modifications that facilitate human liver cell engraftment (Peltz 2013). For example, the TK-NOG mouse expresses a thymidine kinase
transgene within the liver of an immunodeficient mouse strain (Hasegawa et al. 2011), which enables a brief exposure to a non-toxic dose of ganciclovir to induce the rapid and temporally controlled ablation of mouse liver cells. This enables transplanted human liver cells to develop into a mature “human organ” with a 3-dimensional architecture and a gene expression pattern characteristic of mature human liver, which could be stably maintained for >6 months without exogenous drug treatment (Hasegawa et al. 2011). Chimeric TK-NOG mice were shown to be a predictive model for the pattern of human drug metabolism and the occurrence of a human drug-drug interaction for a drug in development (Nishimura et al. 2013), and for identifying human genetic factors affecting drug metabolism (Hu et al. 2013). Since TK-NOG mice do not have ongoing liver toxicity, and do not require treatment with other drugs to suppress their immune system or to prevent liver damage, they could provide an optimal platform for toxicology studies (Peltz 2013). Consistent with this possibility, we recently demonstrated that DILI caused by fialuridurine was easily detected in chimeric TK-NOG mice. (Xu et al. 2014). The humanized livers in TK-NOG mice express mRNAs encoding many human drug transporters (including BSEP) at levels that are equivalent to those in human hepatocytes (Hasegawa et al. 2011). However, we do not know if their biliary tract is functionally humanized, or if they can predict whether a drug will have a human-specific pattern of hepatobiliary clearance. One test drug was previously shown to have a humanized profile of liver clearance in another type of chimeric mouse (Okumura et al. 2007), which suggests that this may be possible. Here, we demonstrate that the human-specific cholestatic toxicity caused by bosentan could have been predicted using chimeric TK-NOG mice.

Methods

Preparation and characterization of chimeric of TK-NOG mice. All animal experiments were performed according to protocols that were approved by the Stanford Institutional Animal Care and Use Committee, and the results are reported according to the ARRIVE guidelines (Kilkenny et al. 2010). TK-NOG mice were obtained from and housed at In Vivo Sciences International (Sunnyvale, CA). TK-NOG mice with humanized livers were prepared using the gancyclovir-conditioning and human hepatocyte transplantation protocol as previously described (Hu et al. 2013). Cryopreserved human hepatocytes were obtained from Celsis In Vitro Inc. (Baltimore, MD). The chimeric mice, the hepatocyte donors and the level of human serum albumin in the humanized mice, which was measured 8 weeks after transplantation, are shown in

Supplemental Table 1. Only chimeric mice having a human plasma albumin level greater than
9 mg/ml was used in this study. Human liver cells were transplanted when the mice were 8 weeks old, and the toxicology studies were performed 8 weeks after transplantation. The plasma human albumin level, which was shown to correlate with the extent of liver humanization, was measured by EIA (Hasegawa et al. 2011).

**Cefmetazole disposition.** Cefmetazole was obtained from Sigma (Cat #: C6086, Sigma, St. Luis MO). For the pharmacokinetic studies, control and humanized TK-NOG mice (n=3) were dosed with Cefmetazole 25 mg/kg IP (Cat: C6086, Sigma, St. Luis MO) IP. Blood samples were collected from the tail vein 0.25, 0.5, 1, 2, 4, and 24 hours after dosing. Bile fluid was collected at 0.5, 4 and 8 hours after dosing using a Vevo 770 image-guided ultrasound system (Visual Sonic, Toronto, Canada). For the drug excretion study, control and humanized mice were dosed with cefametazole 25 mg/kg IP and placed in individual metabolic cages (Hatteras Instruments, Inc. North Carolina). At 8 and 24 hours after dosing; bile, feces and urine were collected for analysis.

**Cefmetazole analysis.** To 5 μL of mouse plasma, urine or bile; 15 μL of cold acetonitrile (containing an internal standard) was added to precipitate protein. The solution was then centrifuged at 14,000 rpm for 5 minutes. Ten μL of supernatant was transferred to an autosampler vial with 90μL water; the solution was vortexed; and 5 μL was injected for analysis. Fecal samples were weighed, and then 20 μL/mg of acetonitrile (with internal standard) was added. The samples were homogenized with Precelly metal beads (2.8mm) in a Precellys 24-dual homogenizer (Bertin Technologies, France).

A Bruker UHPLC system (Bruker Chemical and Applied Markets, Fremont CA) and an YMC-Pack Pro C18 RS, 2.0 x 50mm, 3 μm column (YMC Co.,Ltd, Kyoto, Japan) maintained at 40 °C were used for the analysis. The mobile phase consisted of 0.1% formic acid (FA), 2 mM ammonium formate in water (mobile phase A) and 0.1% FA, 2 mM ammonium formate in methanol (mobile phase B), and gradient elution starting at 20% mobile phase B, keep for 1 minute and ramping with a constant slope to 95% mobile phase B at 2 min, and keep at 95% mobile phase B for 0.5 min at flow rate 0.4mL/min was used with a run time of 7 min including re-equilibration to initial conditions. The injection volume was 5 μL. Cefzolin (Sigma-Aldrich, St. Louis MO) was used as the internal standard. A Bruker EVOQ Elite (Bruker Chemical and Applied Markets, Fremont CA) was utilized with an electrospray ionization source (ESI) and positive polarity. The source parameters were spray voltage of 400 v, cone gas flow of 15 unit,
cone temperature of 300 °C, heated probe gas flow of 40 unit, heated probe temperature of 450 °C, Nebulizer gas flow of 50 unit and exhaust gas on. The mass spectrometer parameters listed in the table below were used for the analysis.

<table>
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<th>Compound Name</th>
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<th>Precursor Ion (m/z)</th>
<th>Product ion (m/z)</th>
<th>Collision Energy (v)</th>
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In situ perfusion. These studies were performed using a modified version of previously described methods (Zamek-Gliszczynski et al. 2006). The mice were fully anesthetized by injection of 0.2 ml per 10 grams body weight of a 1.2% Avertin (2, 2, 2-Tribromoethanol) solution (IP). The abdominal cavity of the anesthetized mouse was opened to expose the intestines, liver, and gallbladder. The common bile duct was ligated above the duodenum to prevent bile from entering the intestine. A loose suture was then placed around the inferior vena cava below the liver, and the portal vein was cannulated with a 24-gauge catheter. The liver was perfused at a rate of 2-5 ml/min with drug-free Krebs-Henseleit buffer (Cat #: K3753, Sigma, St. Luis MO) solution containing 5 μM taurocholate (Cat #: 86339, Sigma, St. Luis MO) that was continuously oxygenated. The portion of the inferior vena cava that was below the liver) was then immediately severed by an incision placed below the suture. Next, the gallbladder was cannulated with PE-10 tubing, and then the superior vena cava was cannulated with a 24-gauge catheter. Then, the loose suture around the inferior vena cava was tied off to direct all perfusate outflow through the cannula inside the inferior vena cava above the liver. After a 10-min pre-perfusion period, which allows for equilibration of the liver temperature and bile flow, the liver was perfused with buffer containing 5 uM CDCF (Cat #: C369, Life technology,Grand island, NY), 5uM CDCF and 20 uM Rifampicin (Cat #: R3501, Sigma, St. Luis MO), or 5 uM CDCF and 20 uM probenecid (Cat #: P8761, Sigma, St. Luis MO). In other in situ perfusion experiments, 56 ug/ml cefmetazole was added to the perfusion buffer. Bile and the outflow perfusate samples were collected at 10 min intervals. At the end of the experiment, the livers were isolated and snap frozen for subsequent analysis. The in situ perfusion data was analyzed using previously described methods (Zamek-Gliszczynski et al. 2006) and the following formulas:
Hepatic extraction ratio \( (E_h) = \frac{C_{\text{reservoirTi}} - C_{\text{perfusateTi}}}{C_{\text{reservoirTi}}} \) 

Hepatic clearance rate \( (\text{Cl}_h) = \text{Perfusion rate} \times E_{hTi} \)

Biliary excretion rate \( (E_b) = \frac{\text{bile [drug](mM)/perfusion rate} \times T}{\text{average } C_{\text{reservoir} \Delta T}} \) 

Biliary clearance rate \( (\text{Cl}_b) = \frac{\text{Biliary excretion rate}}{\text{Liver concentration}} \)

Equilibrium distribution ratio \( (K_p) = \frac{\text{Tissue concentration at steady state}}{\text{Outlet concentration at steady state}} \)

Where \( T_i \) is the time point for the measurement; \( C \) is the concentration in the reservoir or the perfusate; and \( \Delta T \) is the time interval. The tissue concentration was determined by measurement of the bosentan or metabolite concentration in the perfused liver tissue, and the outlet concentration was the drug or metabolite concentration in the perfusate outflow at the last (40 minute) time point. The steady state start points were selected based upon when the drug concentration versus time curve reached its plateau level. All clearance values were normalized for liver mass.

**Bosentan Toxicology Study.** Bosentan was synthesized by Bosche (New Brunswick, NJ); it was shown to be >99% pure by LC/MS analysis, and its chemical structure was confirmed by 2-D NMR analysis. For drug formulation, a 20% Cavasol W7 HP Pharma (Wacker Chemical, München, Germany) was used as the solvent, which we refer to as CAVA. Bosentan powder was first dissolved in 20% CAVA to a concentration of 20 mg/ml, and the pH was adjusted to 7.7-7.9. Control or humanized TK-NOG mice were first treated with bosentan 40, 80 or 160 mg/kg/day by oral gavage. Then control and humanized TK-NOG mice were treated with bosentan 160 mg/kg/day by oral gavage for 28 days. Then, the liver tissue was harvested, and then placed in: 10% formalin for hematoxylin and eosin staining, 4% paraformaldehyde for immunohistochemistry, and OCT for immunofluorescence staining. The plasma levels of total cholesterol, total protein and gamma glutamyltransferase (GGT) were measured using a Heska DryChem 7000 analyzer (HESKA, Loveland, CO) according to the manufacturer’s instructions. In a subsequent study, control and humanized TK-NOG mice were treated with bosentan 32 or 6 mg/kg/day by oral gavage for 14 days.
Statistical analyses. To facilitate comparisons between groups of mice, the data was first logarithm transformed. This enabled the group data being compared to be more homogeneous, and the data distribution was closer to a Gaussian distribution. Then, a two-sample, two-sided t test was applied to test the significance of the observed differences between drug-treated and the corresponding vehicle-treated humanized mice. Finally, when plasma ALT, alkaline phosphatase, GGT, total protein and total Cholesterol levels were investigated simultaneously, all p-values were adjusted using the Benjamini-Hochberg method (Benjamini and Hochberg 1995) to control for the false-discovery rate.

Results

Chimeric TK-NOG mice have a humanized profile of biliary drug clearance. A cephalosporin antibiotic (cefmetazole) has been used to characterize species-specific pathways for drug excretion because it is eliminated in an un-metabolized form. Rodents clear this drug primarily via biliary excretion, while humans clear it by renal excretion (Okumura et al. 2007). Therefore, we compared the cefmetazole clearance pathways in control TK-NOG mice and TK-NOG mice with highly humanized livers (Supplemental Table 1). The plasma human albumin concentrations in the chimeric mice (12.4 ± 2.2 mg/ml), which correlate with the extent of liver humanization (Hasegawa et al. 2011), indicate that the livers were over 90% humanized. The amounts of cefmetazole in urine, feces and bile obtained from control and humanized TK-NOG mice were measured for a 24-hour period after administration of cefmetazole (25 mg/kg IP). The amount of cefmetazole in feces was 8-fold below (p=0.006) and in urine was 5-fold greater (p=0.03) in humanized TK-NOG mice than in control TK-NOG mice (Supplemental Table 2). Cefmetazole had the same plasma pharmacokinetic profile in control and humanized mice, but their biliary profiles were substantially different. The maximal cefmetazole concentration in bile obtained from control TK-NOG mice was 55-fold greater than in humanized TK-NOG mice (p=0.024) (Supplemental Figure 1). In humanized TK-NOG mice, only 18% of this drug was cleared in an unchanged form through the feces (biliary excretion) and 81% was cleared by renal clearance. In contrast, 71% was cleared by biliary excretion and 26% by renal elimination in control TK-NOG mice (Fig. 1). These studies demonstrate that the livers in humanized TK-NOG mice have a humanized profile of biliary cefmetazole excretion.
We wanted to determine if interspecies differences in biliary cefmetazole clearance could be characterized using an *in situ* liver perfusion system (Fig. 2). As described in the supplement, *in situ* perfusion experiments using a fluorescent non-metabolizable dye and transport inhibitors, established that dye uptake into the liver and its transport into bile are saturable and carrier dependent processes in the livers of both humanized and control mice (Supplemental Figure 2). We next examined whether the *in situ* liver perfusion system could be used to characterize the basis for the different profiles of cefmetazole biliary transport in mice with control and humanized livers. To do this, cefmetazole (56 ug/ml) was added to the perfusion buffer, and the amount of cefmetazole in the liver and bile was measured as a function of the time of perfusion. Cefmetazole transport into bile reached its steady state rate after 20 minutes of perfusion, which persisted until the end of the perfusion period (Supplemental Figure 3). The rate of hepatic cefmetazole uptake (i.e. the hepatic extraction ratio or Eh) was low in both control and humanized mice. However, the humanized liver had a 4-fold lower rate (p=0.007) of biliary cefmetazole clearance (CL\(_b\)= 0.02) relative to control mice (CL\(_b\)=0.08), and a five-fold (p=0.003) higher affinity for cefmetazole (Kp=2.7) than control mouse liver (Kp=0.5) (Supplemental Table 3). The perfusion data reveals that the humanized liver has a lower rate of cefmetazole biliary transport and higher cefmetazole retention, which explains why the biliary clearance of cefmetazole is reduced in humans.

**Bosentan-induced liver injury in humanized TK-NOG mice.** We next investigated whether the humanized TK-NOG model could predict whether bosentan would cause liver toxicity in humans. Chimeric and control TK-NOG mice (Supplemental Table 1) were first treated with a bosentan 160 mg/kg/day PO for 28 days, which is a dose that is 23-fold above the total daily human dose (Rubin et al. 2002). This treatment was well tolerated by control and humanized TK-NOG mice; they did not lose weight over the one-month period of treatment (p-value 0.94) (Fig. 3A); there were no deaths; and none became overtly jaundiced. There was a temporary decrease in the weight of the vehicle-treated humanized mice (~ 2 g) after 2 weeks, but their weight returned to the initial level by 4 weeks. Mice often experience a temporary decrease in weight during the early phase of a drug study. This is due to the fact that they are frequently handled, subject to daily weighing, daily injections, and their blood is frequently drawn. However, there was clear serologic evidence of DILI in the bosentan-treated humanized mice; their plasma ALT (875 ± 55.8) was increased 15-fold (p=4.6 x 10\^-6\) over bosentan-treated control (59.8 ± 6.6) TK-NOG mice. The ALT levels in bosentan-treated humanized mice were 6.3-fold increased (p=7 x 10\^-6\) relative to vehicle-treated humanized TK-NOG mice (Fig. 3B).
The serum ALT levels were followed weekly in the bosentan-treated humanized mice; they were elevated after only one week of dosing, and remained elevated throughout the 4-week dosing period.

Analyses of other liver injury markers confirmed that bosentan caused liver toxicity in humanized, but not in control mice. After 28 days of dosing, plasma alkaline phosphatase levels in bosentan-treated humanized TK-NOG mice (508 ± 59) were increased by 7-fold \((p=3.1 \times 10^{-7})\) relative to bosentan-treated control (73 ± 5.2) mice, and were 4-fold above \((p=4.0 \times 10^{-5})\) that of vehicle-treated (123 ± 6.5) humanized TK-NOG mice (Fig. 3C). The plasma GGT levels in bosentan-treated humanized TK-NOG mice (21 ± 0.8) were also increased relative to bosentan-treated control (10 ± 0.8) \((p=8.0 \times 10^{-5})\) or vehicle-treated humanized mice (11 ± 0.8) \((p=7.5 \times 10^{-5})\) (Fig. 2C). Of importance, the serum ALT and alkaline phosphatase levels were not elevated above the upper limit of normal in the bosentan-treated control TK-NOG mice (Figs. 2B-C). Since bosentan reportedly causes hepatic bile acid accumulation via inhibition of BSEP transporter activity, we measured the total bile acid concentration in the plasma of control \((n=3)\) and humanized mice \((n=3)\) after 0, 7 or 28 days of bosentan treatment. After 28 days of bosentan treatment, the total bile acid concentration in plasma obtained from humanized mice (45.1 ± 21.8 uM) was 5-fold higher than on day 0 (8.9 ± 1.6 uM) \((P=0.046)\). In contrast, in the plasma of control mice, there was no change in the total bile acid concentration on days 0 (1.9 ± 0.25 uM) and 28 (3.2 ± 1.1 uM) \((p=0.38)\) (Fig 3D). Other liver function parameters, such as the total protein and cholesterol levels, were within normal limits in all groups of mice (Supplemental Figure 3). Although their average values were slightly reduced in the bosentan-treated humanized mice, there was no significant difference in the total plasma cholesterol \((p=0.20)\) or protein \((p=0.37)\) between the bosentan and vehicle-treated groups of humanized mice. Examination of liver sections obtained from humanized TK-NOG mice revealed no histologic abnormalities except for occasional focal mild fatty change in areas with human hepatocytes, but these were noted in livers obtained from both vehicle and bosentan-treated mice (Supplemental Figure 4). Mouse hepatocytes in all groups of mice were unremarkable. Thus, bosentan-induced liver injury is clearly detected in chimeric (but not control) mice; the laboratory data and histology observed in chimeric mice mimics the cholestatic pattern seen in bosentan-treated human subjects (Fattinger et al. 2001).

To investigate whether this toxicity was dose-dependent, highly humanized chimeric TK-NOG mice were treated with 32 or 6-mg/kg/day oral bosentan for 14 days, before drug dosing was
electively terminated. There were no deaths, and the humanized mice treated with these doses did not lose body weight after 14 days of treatment with either the 32 (P=0.5) or 6 mg/kg/day dose (P=0.08) (Supplemental Figure 5). However, there was clear evidence that bosentan caused liver toxicity in the humanized, but not in control mice. However, after treatment with the 6 or 32 mg/kg/day doses of bosentan, humanized TKNOG mice had statistically significant elevations of plasma ALT (p = 0.006 and 0.003, respectively, Fig. 4A) and alkaline phosphatase (p = 0.002 and 0.003, respectively, Fig. 4B) levels relative to their pre-treatment values. In contrast, neither bosentan dose caused the plasma ALT or alkaline phosphatase levels to increase in control mice, nor were the plasma ALT or alkaline phosphatase levels increased in vehicle-treated chimeric mice. This data establishes that bosentan-induced liver toxicity was dose dependent, and could be detected in chimeric TK-NOG mice treated with a bosentan dose that is only 2-fold above the usual human dose.

Discussion

Our results demonstrate that the biliary tract of chimeric TK-NOG mice is functionally humanized, and that the TK-NOG model can be used to identify drugs that may cause cholestatic liver toxicity in humans. We also demonstrate that the basis for inter-species differences in biliary drug elimination can be characterized by coupling the TK-NOG model with an in situ liver perfusion system. The in situ perfusion results indicate that the inter-species differences in cefmetazole elimination pathways result from a species-specific differences in the rate of biliary drug transport and in liver retention of the drug. Thus, the type of liver cell, irrespective of whether the cells in the biliary ducts are humanized, determines whether cefmetazole is predominantly eliminated by biliary (mouse) or renal pathways (human). Because of this, TK-NOG mice with humanized livers have a humanized profile of cefmetazole elimination, which is substantially different from that of control mice.

Given the significant public health problem caused by DILI, and the difficulties associated with regulatory actions occurring after drug approval (Ostapowicz et al. 2002; Watkins and Seeff 2006), identification of candidate medications that are likely to cause human-specific DILI is of major importance. Bosentan-induced cholestatic toxicity could easily be detected in humanized TK-NOG mice after only one week of dosing, even when treatment of conventional mice for one month provided no evidence of its hepatotoxic potential in humans. At present, we do not understand the mechanism underlying the human-specific susceptibility to bosentan-induced
liver toxicity. Since bosentan is an equipotent inhibitor of bile acid transport by murine and
human BSEP (Mano et al. 2007), this activity alone can not explain the human-specific
hepatotoxicity. It is likely that inter-species differences in bile acid biosynthesis may underlie
their differential susceptibility. Bosentan’s effect on bile transport resembles (in some ways) that
of bile duct ligation, and humans and rodents exhibit very different responses to biliary
obstruction. In mice (Zhang et al. 2012) and rats (Takita et al. 1988; Naito et al. 1996), bile duct
ligation causes a rapid increase in the synthesis of mostly (95%) water-soluble muricholic acids
that are excreted in the urine, which provides rodents with a route for elimination of hepatotoxic
bile acids (Dueland et al. 1991; Naito et al. 1996). In contrast, human liver produces a very
different set of hepatotoxic bile acids (chenodeoxycholic acid, cholic acid and their glycine
conjugates) (Russell 2003), which could be more difficult to eliminate when BSEP is inhibited.
Given their differential susceptibility to bosentan-induced liver toxicity, subsequent experimental
analyses of bile acid production and transport in control and humanized mice could enable us to
understand the increased susceptibility of the human liver to bosentan-induced toxicity.

Despite the bosentan-induced elevations in plasma liver enzymes and in total bile acid
concentration, bosentan induced minimal histologic abnormalities in the livers of chimeric mice.
Bosentan-induced liver toxicity in humans is reversible, and we do not have liver tissue from
bosentan-treated humans for comparison. Hence, we do not know what type of histopathology
to expect in the livers of the humanized mice. Nevertheless, if humanized TK-NOG mice had
been used during the pre-clinical evaluation of bosentan, important information about its
potential to cause cholestatic liver toxicity in humans would have been available to
pharmaceutical companies and to government regulators at an early stage in its development.
We have now demonstrated that the TK-NOG model could identify drugs causing human-
specific DILI by two different mechanisms (mitochondrial-toxic (Xu et al. 2014) and cholestatic),
which indicates that their use in pre-clinical drug assessment could improve drug safety.

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Author contributions
Participated in research design: Guo, Peltz
Conducted experiments: Xu, Wu, Sachiko Nishimura, Toshihiko Nishimura, Takedai Takeda,
Guan, Day, Hillgren
Contributed new reagents or analytic tools: Yang, Yates
Performed data analysis: Guo, Zheng, Michie
Wrote or contributed to the writing of the manuscript: Xu, Peltz
References


Footnotes

Manhong Wu and Dan Xu are co-first authors of this paper

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Legends for Figures

Figure 1. To assess the pattern of cefmetazole clearance, control (n=3) and humanized (n=3) TK-NOG mice were dosed with cefmetazole (25 mg/kg IP), and the amount of cefmetazole in blood, urine and bile was measured by LC/MS analysis. The percentage of cefmetazole in urine, bile or feces collected over a 24-hr period is shown for control and humanized TK-NOG mice. While cefmetazole was primarily eliminated through renal excretion (81%) in humanized mice; it was mostly eliminated via the feces (71%) in control mice.

Figure 2. Diagram of the in situ perfusion system. The liver is continuously perfused with a solution containing 100 ug/ml cefmetazole, which enters the liver via the portal vein. The inferior vena cava (IVC) is ligated; so the perfusate exits via the superior vena cava (SVC) after passing through the liver. The common bile duct is cannulated to direct all bile flow to the gallbladder, which is where the bile is collected.

Figure 3. Control (non-humanized) (n=6) or chimeric (n=6) TK-NOG mice were treated with bosentan 160 mg/kg/day PO or vehicle for 28 days, and their weights were measured on a daily basis (A). The plasma ALT (B), alkaline phosphatase and GGT (C) levels were measured after 4 weeks of dosing. The plasma ALT levels were also measured after 1, and 2 weeks in a (B). In B-C, each bar is the average + SD of 6 measurements in control or humanized TK-NOG mice, and the dashed line indicates the upper limit of normal for the measured parameter. (D) Bosentan treatment induces an increase in plasma total bile acids in humanized TK-NOG mice. Plasma was collected from both control (n=3) and humanized (n=3) TK-NOG mice after 0 and 28 days of treatment with bosentan (160 mg/kg/d). After 28 days of bosentan treatment, the total plasma bile acid concentration in humanized mice was increased 5-fold (P=0.046), while in control mice there was no change in the plasma total bile acid concentration (p=0.38).

Figure 4. Control (n=6) or chimeric TK-NOG mice (n=6 per group) were treated with 32 or 6 mg/kg/day PO Bosentan or vehicle (n=3) for 14 days, and their plasma ALT (A) and alkaline phosphatase (B) levels were measured on day 0, 7 and 14. Each bar is the average + SD of the measurements in control or humanized TK-NOG mice, and the dashed line indicates the upper limit of normal for the measured parameter.
Figure 1

Humanized TK-NOG

- Urine: 81%
- Feces: 18%
- Bile: 1%

Control TK-NOG

- Urine: 26%
- Feces: 71%
- Bile: 3%
Figure 3D

Total Bile Acid (μmol/L)

- day 0
- day 28

control  
Hu
Figure 4B

**ALK Phos (U/L)**

**6 mg/kg/day**

Day 0: Control (71), Veh Hu (129), Bosentan Hu (140)

Day 7: Control (77), Veh Hu (135), Bosentan Hu (262)

Day 14: Control (68), Veh Hu (127), Bosentan Hu (315)

**32 mg/kg/day**

Day 0: Control (84), Veh Hu (129), Bosentan Hu (140)

Day 7: Control (99), Veh Hu (135), Bosentan Hu (383)

Day 14: Control (72), Veh Hu (127), Bosentan Hu (393)