Open access • Journal Article • DOI:10.1038/CLPT.2013.143

Association of CYP2C9*2 with bosentan-induced liver injury. — Source link

Svetlana Markova, T. De Marco, Nasrine Bendjilali, Erin Kobashigawa ...+13 more authors

Institutions: University of California, San Francisco, Genentech, University of Washington

Published on: 01 Dec 2013 - Clinical Pharmacology & Therapeutics (Clin Pharmacol Ther)

Topics: Bosentan, Alanine transaminase and Endothelin receptor antagonist

Related papers:

- The endothelin antagonist bosentan inhibits the canalicular bile salt export pump: a potential mechanism for hepatic adverse reactions.
- Clinical Pharmacology of Bosentan, a Dual Endothelin Receptor Antagonist
- Causality assessment of adverse reactions to drugs--I. A novel method based on the conclusions of international consensus meetings: application to drug-induced liver injuries.
- Incidence, Presentation, and Outcomes in Patients With Drug-Induced Liver Injury in the General Population of Iceland
- Incidence of drug-induced hepatic injuries: a French population-based study.

Share this paper:

View more about this paper here: https://typeset.io/papers/association-of-cyp2c9-2-with-bosentan-induced-liver-injury-1sviieiyqa

UCSF

UC San Francisco Previously Published Works

Title

Association of CYP2C9*2 with bosentan-induced liver injury.

Permalink

https://escholarship.org/uc/item/9275r6mk

Journal

Clinical pharmacology and therapeutics, 94(6)

ISSN

0009-9236

Authors

Markova, SM
De Marco, T
Bendjilali, N
et al.

Publication Date

2013-12-01

DOI

10.1038/clpt.2013.143

Peer reviewed

eScholarship.org Powered by the California Digital Library
University of California

see COMMENTARY page 629

Association of *CYP2C9**2 With Bosentan-Induced Liver Injury

SM Markova[1], T De Marco[2], N Bendjilali[3], EA Kobashigawa[2], J Mefford[4], J Sodhi[5], H Le[5], C Zhang[5], J Halladay[5], AE Rettie[6], C Khojasteh[5], D McGlothlin[2], AHB Wu[7], W-C Hsueh[3], JS Witte[4], JB Schwartz[8] and DL Kroetz[1]

Bosentan (Tracleer) is an endothelin receptor antagonist prescribed for the treatment of pulmonary arterial hypertension (PAH). Its use is limited by drug-induced liver injury (DILI). To identify genetic markers of DILI, association analyses were performed on 56 Caucasian PAH patients receiving bosentan. Twelve functional polymorphisms in five genes (*ABCB11*, *ABCC2*, *CYP2C9*, *SLCO1B1*, and *SLCO1B3*) implicated in bosentan pharmacokinetics were tested for associations with alanine aminotransferase (ALT), aspartate aminotransferase (AST), and DILI. After adjusting for body mass index, *CYP2C9*2* was the only polymorphism associated with ALT, AST, and DILI ($\beta = 2.16$, $P = 0.024$; $\beta = 1.92$, $P = 0.016$; odds ratio 95% CI = 2.29–∞, $P = 0.003$, respectively). Bosentan metabolism by CYP2C9*2 *in vitro* was significantly reduced compared with CYP2C9*1 and was comparable to that by CYP2C9*3. These results suggest that *CYP2C9*2* is a potential genetic marker for prediction of bosentan-induced liver injury and warrants investigation for the optimization of bosentan treatment.

Pulmonary arterial hypertension (PAH) is a progressive debilitating condition characterized by increased resistance in the pulmonary arterial circulation. Clinically, PAH is defined as a resting mean pulmonary artery pressure ≥25 mm Hg with a normal pulmonary venous pressure ≤15 mm Hg.[1] PAH may have diverse etiology but a common pathogenesis that results in pulmonary vascular remodeling with proliferation and hypertrophy of pulmonary arterial smooth muscle cells, vasoconstriction, endothelial dysfunction, and thrombosis *in situ*.[1] Several vascular changes are associated with PAH, including downregulation of prostacyclin synthase[2] resulting in a relative deficiency of prostacyclin (PGI_2),[3] overexpression of endothelin-1,[4] and downregulation of nitric oxide production with a decrease in intracellular cyclic guanosine monophosphate expression.[5]

Although prognosis for PAH remains poor, major improvements have been made in disease management with the introduction of therapeutic agents targeting various affected pathways.[6] Current treatments of PAH include parenteral prostanoids (epoprostenol, treprostinil, and ilioprost), oral endothelin receptor antagonists (bosentan, sitaxentan, and ambrisentan), and oral inhibitors of phosphodiesterase-5 (sildenafil and tadalafil).[1] To date, there is no compelling evidence for one class of drugs being superior over another, but it is generally accepted that prostanoids, which are difficult to administer and maintain, are used in patients with severe disease or in those who have failed to respond to other therapies. Therefore, oral endothelin receptor antagonists and phosphodiesterase-5 inhibitors often become a first-line treatment in mild-to-moderate PAH.

Bosentan is an oral dual endothelin receptor antagonist approved for treatment of PAH patients with World Health Organization class II–IV symptoms. Bosentan is usually initiated at a dose of 62.5 mg twice daily and increased to 125 mg twice daily after 4 weeks of treatment. The pharmacokinetics of bosentan has been well described.[7] After oral administration, bosentan is absorbed with an absolute bioavailability of 50%, reaching peak plasma concentrations in about 3–4 h. Bosentan is taken up from the blood into the liver by organic anion-transporting polypeptide (OATP)1B1 and OATP1B3,

[1]Department of Bioengineering and Therapeutic Sciences, Institute for Human Genetics, University of California, San Francisco, San Francisco, California, USA; [2]Department of Cardiology, University of California, San Francisco, San Francisco, California, USA; [3]Department of Medical Genetics, Institute for Human Genetics, University of California, San Francisco, San Francisco, California, USA; [4]Department of Epidemiology and Biostatistics, Institute for Human Genetics, University of California, San Francisco, San Francisco, California, USA; [5]Department of Drug Metabolism and Pharmacokinetics, Genentech, South San Francisco, California, USA; [6]Department of Medicinal Chemistry, University of Washington, Seattle, Washington, USA; [7]Department of Laboratory Medicine, University of California, San Francisco,, San Francisco, California, USA.; [8]Department of Medicine, University of California, San Francisco, and the Jewish Home, San Francisco, California, USA., Correspondence: DL Kroetz (deanna.kroetz@ucsf.edu)

Received 24 April 2013; accepted 1 July 2013; advance online publication 18 September 2013. doi:10.1038/clpt.2013.143

and metabolized by cytochrome P450 isozymes(CYP)3A4 and CYP2C9 to three major metabolites, one of which (Ro 48–5033) may contribute to up to 20% of total drug response. Bosentan is mainly cleared by hepatic metabolism, with an elimination half-life of approximately 4–5 h. Bosentan has been shown to significantly improve exercise ability and reduce the rate of clinical worsening in PAH patients.[8,9] Unfortunately, exposure-dependent liver toxicity, manifesting in the form of increased activity of aminotransferases in 10–12% of treated patients,[9,10] limits bosentan use.

The possible mechanisms of bosentan-induced liver injury include a decrease in hepatic uptake of bosentan by OATP1B1/1B3, the subsequent increase in systemic exposure[11,12] as well as a decrease in activity of CYP enzymes, and the subsequent increase in both systemic and intrahepatic exposure.[13,14] There is also evidence that bosentan inhibits hepatic canalicular efflux transporters, such as the bile salt export pump (BSEP) and multidrug resistance–associated protein 2 (MRP2), which are responsible for bile acid excretion, leading to intrahepatic bile acid accumulation and consequently liver damage.[15–17] The latter mechanism might be independent or sequential to increased bosentan exposure. The majority of studies have focused on drug–drug interactions resulting in inhibition of liver uptake transporters or CYP enzymes. To date, no attempts have been made to study the influence of naturally occurring reduced-function single-nucleotide polymorphisms (SNPs) in genes encoding the proteins involved in bosentan disposition (CYP2C9, CYP3A4, OATP1B1/1B3, BSEP, and MRP2).

The current study was designed to examine associations between bosentan-induced liver injury and reduced-function SNPs in genes encoding CYP2C9, CYP3A4, OATP1B1, OATP1B3, BSEP, and MRP2. Putative genetic markers of bosentan-induced liver injury could be further investigated as biomarkers for stratification of PAH patients for appropriate treatment regimens.

RESULTS

Cohort analysis

The original study cohort consisted of 92 ethnically diverse PAH patients. The majority of patients were Caucasians (61%), with smaller numbers of Asians (15%), Hispanics (15%), and African Americans (5%). There was no association of race with either change in alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels or DILI (data not shown). Only the Caucasian group ($n = 56$) was large enough for genetic association analysis. Characteristics of the Caucasians included in the current analysis are shown in **Table 1**. The cohort, consisting mostly of women (71%), had an average age of 51 ± 13 years and mean body mass index of 30.8 ± 7.6 kg/m^2. Less than 10% of the patients had a history of previous liver disease. The range of cumulative bosentan exposure was large, reflecting interpatient differences in the time of bosentan therapy. More than half of the patients (57.1%) were coadministered an OATP1B1 inhibitor (sildenafil in most cases). Comedications that were known inhibitors of CYP2C9, CYP3A4, and MRP2 were used in 5.3%, 8.9%, and 3.6% of patients, respectively.

Table 1 Anthropometric, physiologic, and clinical characteristics of Caucasian pulmonary arterial hypertension study population

Number of patients	56
Age (years)	51 ± 13[a]
Sex M:F (%)	29:71
BMI (kg/m^2)	30.8 ± 7.6
History of liver disease (%)	8.9
Bosentan exposure (g/m^2)	611 ± 677
CYP2C9 inhibitors (%)	5.3
CYP3A4 inhibitors (%)	8.9
MRP2 inhibitors (%)	3.6
BSEP inhibitors (%)	0
OATP1B1 inhibitors (%)	57.1
OATP1B3 inhibitors (%)	0

BMI, body mass index; BSEP, bile salt export pump; CYP, cytochrome P450; F, female; M, male; MRP2, multidrug resistance-associated protein 2; OATP, organic anion–transporting polypeptides.

[a]Data are presented as either mean ± SD or percentage of total group.

Associations between candidate SNPs and increases in ALT and AST activity in Caucasian PAH patients treated with bosentan

In a univariate linear regression analysis, body mass index showed a trend for negative correlation with ALT and AST increase ($\beta = 0.96$, $P = 0.06$; $\beta = 0.98$, $P = 0.13$, respectively). A single SNP in *CYP2C9* (*CYP2C9**2, rs1799853) showed a trend for association with ALT and AST increase in univariate analysis (**Table 2**) and was further tested in multivariate analysis. In a multivariate model adjusted for body mass index, *CYP2C9**2 remained significantly associated with increases in ALT ($\beta = 2.16$, $P = 0.024$; **Figure 1a**) and AST ($\beta = 1.92$; $P = 0.016$; **Figure 1b**) activity.

Associations between candidate SNPs and DILI in Caucasian PAH patients treated with bosentan

In univariate logistic regression (continuous variables) or by Fisher's exact test (dichotomous variables), none of the clinical covariates showed any trend for association with occurrence of DILI (**Table 3**), whereas a SNP in *SLCO1B1* (rs11045819) showed a trend for association with bosentan-induced liver injury (**Table 3**). *CYP2C9**2 was the only polymorphism significantly associated with occurrence of DILI (odds ratio 95% confidence interval = 2.29–∞; $P = 0.003$; **Table 3** and **Figure 2**). Multivariate analysis was not performed due to the small sizes of the cohort and the DILI group.

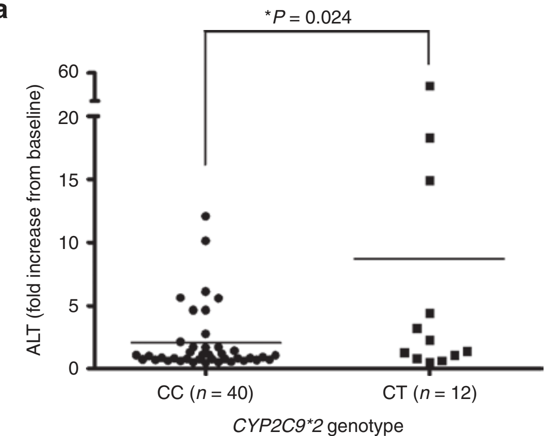
In vitro bosentan metabolite identification and functional characterization of CYP2C9*1, CYP2C9*2, and CYP2C9*3 enzymes in bosentan metabolism

Bosentan metabolic pathways were confirmed as described previously.[7] Bosentan is metabolized to Ro 48–5033 through hydroxylation and to Ro 47–8634 through *O*-demethylation. Ro 47–8634 is further hydroxylated to Ro 64–1056, and Ro 48–5033 is subsequently metabolized through *O*-demethylation

Table 2 Genetic association analysis for ALT and AST increase in 56 pulmonary arterial hypertension patients treated with bosentan

	ALT[a]		AST[a]	
	Fold change[b]	*P*	Fold change[b]	*P*
Age	1.01	0.53	1.01	0.28
Sex	1.15	0.67	1.12	0.68
BMI	0.96	0.06	0.98	0.13
Hx liver disease	0.87	0.78	1.07	0.87
Bosentan exposure	1.00	0.85	1.00	0.81
Comedications	1.01	0.96	0.90	0.69
ABCC2				
rs717620	0.92	0.74	1.04	0.76
rs2273697	0.92	0.78	0.81	0.34
rs1885301	0.94	0.73	1.04	0.82
rs7910642	1.49	0.33	1.08	0.82
rs2804402	1.07	0.73	0.98	0.82
ABCB11				
rs2287622	1.19	0.39	1.01	0.92
SLCO1B1				
rs11045819	1.31	0.39	1.31	0.27
rs2306283	1.16	0.53	1.01	0.99
rs4149056	1.09	0.76	0.84	0.48
SLCO1B3				
rs7311358	1.28	0.38	1.15	0.52
CYP2C9				
*2/rs1799853	2.18	0.026	1.94	0.015
*3/rs1057910	0.61	0.21	0.71	0.28

ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index.
[a]Univariate linear regression was performed, and variants with a *P* value ≤ 0.2 were carried into the multivariate regression analysis adjusted for BMI. The results of multivariate regression are presented in the text. [b]Fold change in ALT and AST was not normally distributed and was natural log transformed for analysis. Coefficients are absolute fold change values. The regression coefficients and the standard errors from the transformed data are presented in **Supplementary Table S3** online.



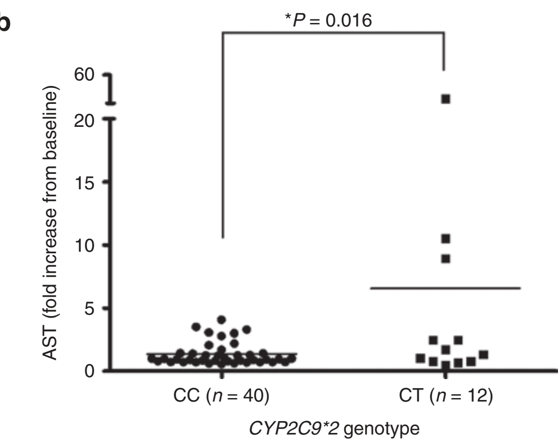


Figure 1 Association between *CYP2C9**2 and changes in (**a**) ALT and (**b**) AST levels in Caucasian PAH patients treated with bosentan. Data are presented as mean fold increase from baseline and were analyzed in a multivariate model corrected for body mass index (BMI); the *P* value is indicated. ALT, alanine aminotransferase; AST, aspartate aminotransferase; PAH, pulmonary arterial hypertension.

to form Ro 64–1056. In preliminary experiments, it was confirmed that 17, 61, and 11% of bosentan is metabolized in 60 min by human liver microsomes (HLMs), rCYP3A4, and rCYP2C9, respectively. All above-mentioned metabolites were formed in HLMs and CYP3A4 reactions, whereas Ro 48–5033 was the only metabolite formed in CYP2C9 reactions (see **Supplementary Table S1** online). Other tested rCYP enzymes (CYP1A2, CYP2B6, CYP2C8, CYP2C19, and CYP2D6) were not implicated in bosentan metabolism (see **Supplementary Table S1**).

Functional activities of CYP2C9*1 (reference), CYP2C9*2, and CYP2C9*3 enzymes in metabolism of bosentan to Ro 48–5033 were evaluated using recombinant enzymes. Both CYP2C9*2 and CYP2C9*3 variants had moderately reduced Ro 48–5033 formation, as compared with CYP2C9*1, at all three bosentan concentrations. In particular, ~2.3% of bosentan was converted to Ro 48–5033 by CYP2C9*2, ~1.8% by CYP2C9*3, and ~2.8% by CYP2C9*1 (**Figure 3a**).

Assessment of hepatotoxicity of bosentan and its major metabolite, Ro 48–5033, was performed in human cultured hepatocytes in the presence and absence of 1-aminobenzotriazole, a pan-CYP450 inhibitor. In the absence of 1-aminobenzotriazole, bosentan and its major metabolite were quickly metabolized, and no hepatocyte toxicity was observed (data not shown). In the presence of 1-aminobenzotriazole, bosentan exhibited hepatotoxicity with an estimated half-maximal inhibitory concentration of ~ 54 μmol/l, whereas its major metabolite had no significant effect on hepatocyte viability at the tested concentrations (**Figure 3b**).

Bosentan uptake by OATP1B1 reference and Pro155Thr (*SLCO1B1* rs11045819) stably transfected HEK293 cells

Bosentan uptake was significantly greater in HEK293 cells stably expressing the OATP1B1 reference transporter as compared with cells expressing empty vector (**Figure 4**). This difference in uptake gradually disappeared with increasing bosentan concentrations from 10 μmol/l to 100 μmol/l (data shown in part in

Table 3 Genetic association analysis for drug-induced liver injury in 56 pulmonary arterial hypertension patients treated with bosentan

	OR[a]	OR 95% CI lower limit	OR 95% CI upper limit	*P*
Age	0.94	0.86	1.04	0.24
Sex	1.21	0.09	68.2	1.00
BMI	0.96	0.83	1.11	0.62
Hx liver disease	0.00	0.00	18.2	1.00
Bosentan exposure	0.99	0.99	1.00	0.39
Comedications	0.58	0.04	8.61	0.62
ABCC2				
rs717620	1.59	0.11	23.5	0.64
rs2273697	0.46	0.01	6.18	0.64
rs1885301	1.86	0.14	103	1.00
rs7910642	0.00	0.00	8.41	1.00
rs2804402	0.28	0.02	4.21	0.23
ABCB11				
rs2287622	0.45	0.03	6.74	0.59
SLCO1B1				
rs11045819	4.60	0.30	71.5	0.17
rs2306283	2.01	0.15	112	1.00
rs4149056	0.54	0.01	7.26	1.00
SLCO1B3				
rs7311358	0.83	0.02	11.2	1.00
CYP2C9				
*2/rs1799853	∞	2.29	∞	0.003
*3/rs1057910	0.00	0.00	8.41	1.00

BMI, body mass index; CI, confidence interval; OR, odds ratio; PAH, pulmonary arterial hypertension.

[a]Odds ratio was calculated by logistic regression for continuous variables and by Fisher's exact test for dichotomous variables.

Figure 4). Bosentan transport by the OATP1B1 Pro155Thr variant was similar to that of the reference transporter (Figure 4).

DISCUSSION

Bosentan significantly improves manageability and prognosis in PAH patients,[1,8,9] but unfortunately, exposure-dependent liver toxicity manifesting in the form of elevated aminotransferases in 10–12% of treated patients[9,10] limits its use. The mechanisms of bosentan-induced liver injury have been intensively studied in the past decade. Bosentan-induced liver injury has been attributed at least in part to the accumulation of bile acids in hepatocytes as a result of inhibition of BSEP by bosentan.[16–18] BSEP is an adenosine-5'-triphosphate–dependent transmembrane transporter located on the canalicular membrane of hepatocytes. It pumps bile acids from hepatocytes into the bile; therefore, inhibition of its function results in cholestasis.[19] However, in *in vitro* studies, bosentan produced inhibitory effects on BSEP at much higher (50–100 μmol/l)[16–18] than clinically observed plasma concentrations (peak concentration: ~2–4 μmol/l).[7] This

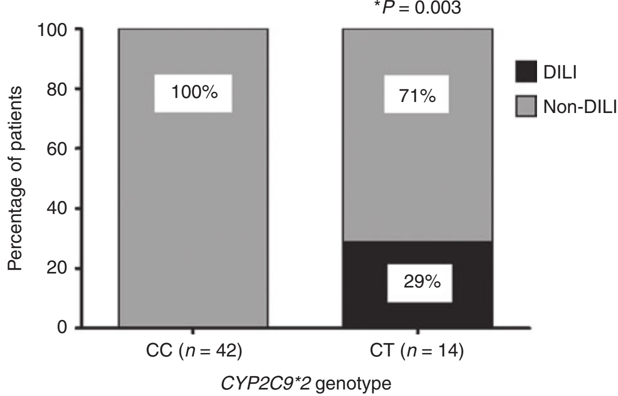
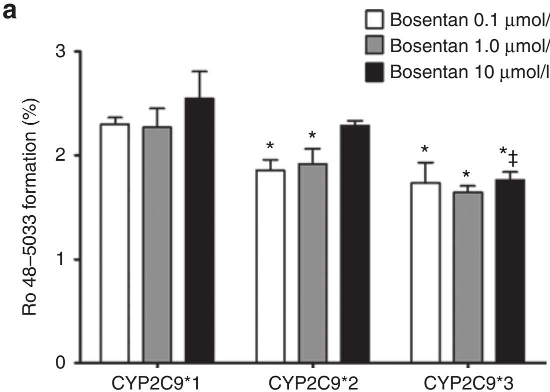


Figure 2 Association between *CYP2C9*2* and drug-induced liver injury (DILI) in Caucasian PAH patients treated with bosentan. Data are presented as percentage of DILI and non-DILI patients in each allele group and were analyzed using Fisher's exact test; the *P* value is indicated. PAH, pulmonary arterial hypertension.



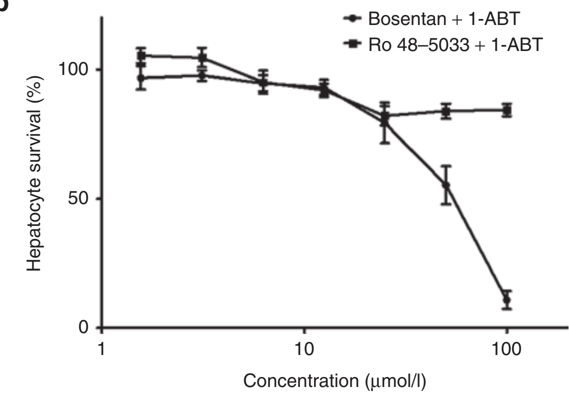


Figure 3 Bosentan metabolism by CYP2C9 variant proteins and CYP-dependent hepatotoxicity. (**a**) rCYP2C9*1 (reference), rCYP2C9*2, and rCYP2C9*3 were incubated with 0.1, 1, and 10 μmol/l bosentan for 0 and 60 min, and formation of Ro 48–5033 was quantified by liquid chromatography–tandem mass spectrometry. Data are presented as percentage of bosentan converted to Ro 48–5033 ± SD ($N = 3$). *$P < 0.05$ for difference between CYP2C9 reference and variants; ‡$P < 0.05$ for difference between CYP2C9*2 and CYP2C9*3. (**b**) Hepatocytes preincubated with 1-aminobenzotriazole were treated with increasing concentrations (0–100 μmol/l) of bosentan or its metabolite Ro 48–5033 for 48 h. Cell viability was assessed by measuring cellular adenosine-5'-triphosphate and glutathione levels. Data are presented as percentage of vehicle control. Half-maximal inhibitory concentration (IC_{50}) values were calculated using GraphPad Prism software.

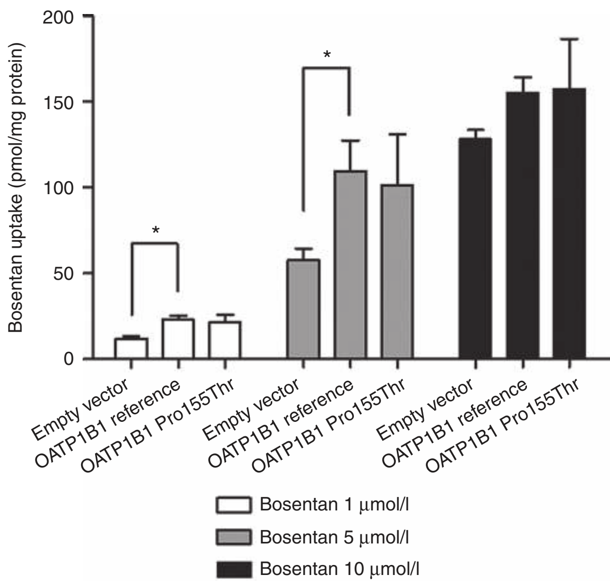


Figure 4 Bosentan transport by OATP1B1 reference and Pro155Thr variant transporters. Empty vector, OATP1B1 reference, and OATP1B1 Pro155Thr–expressing cells were incubated with 1, 5, and 10 µmol/l bosentan for 5 min. Bosentan uptake was measured and normalized to protein levels. Data are presented as mean ± SD ($N = 3$); $P < 0.05$.

suggests possible effects of additional pharmacokinetic factors, which may lead to increased bosentan exposure. Another suggested mechanism of bosentan-induced liver injury involves alterations in MRP2-mediated bile salt formation. MRP2 is also an adenosine-5'-triphosphate–dependent transmembrane transporter located on the canalicular membrane of hepatocytes and is largely involved in the efflux of bilirubin, glutathione, and glucuronide-conjugated compounds into bile. It has been shown that bosentan stimulates MRP2 function,[15,17] causing an increase in bilirubin and glutathione secretion, which results in intermittent uncoupling of lipids from bile salt secretion.[15] Phospholipids normally reduce the cytotoxicity of bile salts; thus, uncoupling of lipids from bile salt secretion increases bile salt toxicity and may contribute to liver damage.

On the basis of the data implicating MRP2 and BSEP in bosentan disposition, we hypothesized that decreased-function variants in BSEP might increase a patient's risk of bosentan-induced liver damage, whereas reduced-function MRP2 variants should afford protection against this toxicity. The common *ABCB11* polymorphism rs2287622, encoding the V444A variant of BSEP, was previously shown to be associated with reduced hepatic BSEP expression[20] and drug-induced cholestasis but not with drug-induced hepatocellular injury.[21] *ABCB11* variants may also have a role in intrahepatic cholestasis of pregnancy, although this is controversial.[22,23] In the current study, an association of rs2287622 with the development of bosentan-induced liver toxicity was not observed, possibly due to the small sample size. However, considering that bosentan-induced liver injury is more hepatocellular in nature,[24] perhaps it is not surprising that *ABCB11* variants did not contribute to risk of this toxicity.

The *ABCC2* promoter variant rs717620 has been previously shown to be associated with diclofenac-induced hepatotoxicity, which is similar in presentation to bosentan-induced liver injury.[25] *ABCC2* haplotypes containing rs717620 were also shown to be associated with hepatocellular injury from herbal compounds.[26] Functional data on *ABCC2* promoter variants suggest only modest effects on ABCC2 expression,[27] and there is no consistent report of altered transport by the V417I MRP2 variant (rs2273697).[28,29] In the current study, *ABCC2* variants were not implicated in bosentan-induced liver injury, suggesting involvement of other factors.

Bosentan-induced liver injury has also been attributed to mechanisms leading to increased exposure. Inhibition of proteins involved in metabolism of bosentan (CYP3A4 and CYP2C9) and/or its uptake into the liver (OATP1B1 and OATP1B3) results in increased systemic exposure to bosentan and a higher frequency of bosentan-associated adverse effects. Inhibitors of CYP3A4 (ketoconazole, itraconazole, and ritonavir) and CYP2C9 (fluconazole and amiodarone), as well as inhibitors of OATP1B1/1B3 (cyclosporin A, sildenafil, and ritonavir), increase the peak concentration and area under the concentration–time curve of bosentan in healthy volunteers.[7,14,30] CYP3A4 accounts for 60% and CYP2C9 for 40% of bosentan metabolism in hepatic microsomes,[7] and reduction in their functionality might be critical in the development of liver damage. Assays performed *in vitro* indicated that hepatotoxicity is attributable to bosentan itself (half-maximal inhibitory concentration: ~ 54 µmol/l) and not to its metabolites (**Figure 3b**). The transporters OATP1B1 and OATP1B3 are expressed on the basolateral membrane of hepatocytes and play a major role in transporting bosentan into hepatocytes.[11,18] Loss of their functionality increases systemic bosentan exposure and may also influence nonhepatic adverse effects. On the basis of the data strongly supporting involvement of CYP3A4, CYP2C9, OATP1B1, and OATP1B3 in bosentan pharmacokinetics, it was hypothesized that reduced-function SNPs in these genes will confer higher risk of developing bosentan-induced liver injury.

*CYP2C9*2* is a common polymorphism associated with a poor metabolizer phenotype. This allele encodes the R144C variant of CYP2C9, which has ~50% reduced function.[31] *CYP2C9*2* has been implicated in reduced clearance of warfarin,[32] phenytoin,[33] celecoxib,[34] and losartan,[35] and *CYP2C9**2 genotyping is commonly used in determining initial warfarin dose.[32] The current data suggest that it is the single most important factor influencing the risk of bosentan-induced hepatic injury. Additional studies will be required to validate its use as a biomarker for identifying patients at increased risk of liver toxicity from bosentan treatment. Considering the allele distribution in major populations, *CYP2C9**2 will be most relevant in the Caucasian and Hispanic populations, where the minor allele frequency for this variant is 10–15%, as compared with African-American and Asian populations, where it is rarely found (4% and 0% minor allele frequencies, respectively).[36] A preliminary replication in the Hispanic population shows a trend toward association of this SNP with elevated ALT/AST levels (ALT: $\beta = 4.01$, $P = 0.084$; AST: $\beta = 2.48$, $P = 0.154$).

*CYP2C9*3* encodes the I359L CYP2C9 variant, which is characterized by almost complete loss of function.[31,36] This

polymorphism is less common, having a minor allele frequency of 7, 2, and 3% in Caucasian, African-American, and Asian populations, respectively.[36] It has also been implicated in reduced clearance of warfarin,[32] phenytoin,[33] celecoxib,[34] and losartan.[35] In this study, *CYP2C9*3* did not show an association with bosentan-induced ALT or AST increase or bosentan-induced liver injury, although this might not be evident due to sample size limitations. Post hoc power calculations for each SNP confirm the limited power of detecting an association with *CYP2C9**3 and other SNPs with relatively low minor allele frequencies (see **Supplementary Table S3** online). It is plausible that the lack of an association of bosentan liver injury with *CYP2C9**3 could also be due to differential metabolism of bosentan by the variants, but this is not supported by the similar reductions in bosentan metabolism with CYP2C9*2 and CYP2C9*3 recombinant proteins. We also considered the possibility that *CYP2C9**2 is not causative but is tagging a regulatory SNP in the genomic region. *CYP2C9* promoter polymorphisms become of greater importance when induction of CYP2C9 plays a substantial role in a drug's pharmacokinetics.[37] Bosentan induces CYP2C9 at therapeutic concentrations[7] through activation of the pregnane X receptor.[38] Of note, *CYP2C9**2 is in linkage disequilibrium (R^2 = 0.57) with rs2185570 located downstream of *CYP2C9*. This SNP is located in a transcription factor–binding region and DNaseI hypersensitivity cluster, as confirmed by chromatin immunoprecipitation–linked DNA sequencing (ChiP-Seq) and DNase I hypersensitive sites sequencing (DNase-Seq) data from ENCODE, and it has been associated in a genome-wide association study with dehydroepiandrosterone sulfate levels. There is no evidence that CYP2C9 is directly involved in dehydroepiandrosterone or dehydroepiandrosterone sulfate metabolism; however, dehydroepiandrosterone is a known CYP2C9 inducer.[39] *CYP2C9**2 is also in linkage disequilibrium (R^2 = 0.462) with an intronic SNP (rs4086116), that has been associated with warfarin[40] and acenocumarol dosing.[41] Further studies will be required to determine whether regulatory SNPs in the *CYP2C9* genomic region might be contributing to bosentan-induced liver injury.

Several common polymorphisms in *SLCO1B1* have been associated with reduced transporter function, altered pharmacokinetics, and increased risk of substrate-induced toxicity.[42] The *SLCO1B1* polymorphism encoding the OATP1B1 Pro155Thr variant (rs11045819) has been associated with gallstone formation[43] and enhanced lipid-lowering activity of fluvastatin in elderly hypercholesterolemic patients.[44] In the present study, *SLCO1B1* polymorphisms were not significantly associated with ALT or AST increase, although rs11045819 showed a trend toward an association with DILI (**Table 3**). Functional studies confirmed that bosentan is transported by OATP1B1, but there was no evidence for reduced function of this variant (**Figure 4**). At higher bosentan concentrations, OATP1B1 transport was not efficient, suggesting that there might be another transporter(s) involved. Recent studies suggest that OATP1B3 has lower affinity but higher capacity for bosentan transport as compared with OATP1B1.[11] Therefore, OATP1B3 might be responsible for this dose-dependent phenomenon in our cell model. Functional variants also exist for *SLCO1B3*,[45,46] but the current analysis found no evidence that these are important for risk of bosentan-induced hepatic injury. The lack of associations with *SLCO1B1* and *SLCO1B3* variants is perhaps not surprising because reduced-function variants would increase systemic exposure and more likely manifest as nonhepatic toxicities.

In summary, *CYP2C9**2 was identified as a potential risk factor for bosentan-induced liver injury. Additional studies are needed to elucidate the molecular basis of this association because effects on bosentan metabolism are modest. The implications of this finding will require validation in additional patients. If validated, this pharmacogenetic marker might be useful in the identification of patients at increased risk of this serious toxicity and lead to alterations in treatment that would avoid this unnecessary risk.

METHODS

Study population. Human investigations were approved by the local committee on human research, and all patients gave informed consent to participate. A total of 92 ethnically diverse PAH patients at the University of California, San Francisco Medical Center's Pulmonary Hypertension Clinic were recruited into the study during 2003–2008. Medical, social, and laboratory data, including age, sex, anthropometric parameters, smoking status, and history of liver disease, were collected at the time of enrollment. A blood sample was collected for genotyping. Treatment with 125–250 mg bosentan daily was initiated and continued for variable time periods depending on the time of enrollment and/or withdrawal from the study due to development of bosentan-induced liver toxicity. Comedications included phosphodiesterase-5 inhibitors, prostanoids, calcium channel blockers, anticoagulants, diuretics, statins, antifungals, and antimicrobial agents. Activities of serum ALT and AST were measured before initiating bosentan treatment and then monthly to monitor liver function.

Clinical study design. This pharmacogenetic study was designed as a prospective cohort study. First, clinical covariates were defined; these included age, sex, body mass index, history of liver disease, bosentan exposure (cumulative dose in mg/m^2 of body surface area), and comedications that are known inhibitors of CYP3A4 (ritonavir and verapamil), CYP2C9 (fluconazole and metronidazole), OATP1B1 (sildenafil and statins), OATP1B3 (statins and valsartan), MRP2 (ritonavir and statins), and BSEP (rifampicin). Next, genetic covariates were selected. A literature search for reduced-function SNPs was focused on six genes (*CYP3A4*, *CYP2C9*, *SLCO1B1*, *SLCO1B3*, *ABCB11*, and *ABCC2*) encoding proteins involved in bosentan pharmacokinetics. A total of 15 SNPs were selected (see **Supplementary Table S2** online).

Two types of outcomes for testing genetic associations were established. Outcome 1 was defined as the fold increase in ALT and AST activities from time of enrollment to the end of the study or to the time of bosentan discontinuation due to liver toxicity. Outcome 1 was expressed as a continuous variable, logarithmically transformed for analysis. Outcome 2 was classification of DILI, defined as a threefold increase of either ALT or AST above the upper limit of normal at any point in the study (n = 4). Outcome 2 was expressed as a binary variable. In addition to classifying DILI based on liver enzyme elevations, the data were evaluated with the Roussel Uclaf Causality Assessment Method (RUCAM).[47,48] RUCAM was performed by two independent individuals, and all Caucasian cases previously classified as DILI cases on the basis of liver enzyme elevations received RUCAM scores of "possible (n = 3)" or "probable (n = 1)," increasing confidence in the assignment of cases. Data on rechallenges were largely absent. One clinical team cared for these pulmonary hypertension patients; initially, two patients were rechallenged, and both redeveloped an elevation in liver function test. Based on knowledge that DILI was a recognized complication of the drug, rechallenges were

discontinued with the arrival of the clinical availability of a newer agent with reduced risk of liver injury.

Genotyping. DNA samples from 92 PAH patients were extracted and genotyped on the DMET chip (Affimetrix, DMET Early Access Program). The following SNPs were extracted for analysis: *CYP2C9* (*CYP2C9**2/rs1799853 and *CYP2C9**3/rs1057910), *SLCO1B1* (rs11045819, rs2306283, and rs4149056), *SLCO1B3* (rs414917 and rs7311358), *ABCB11* (rs2287622), and *ABCC2* (rs717620, rs2273697, rs8187692, and rs17222723). In addition, *ABCC2* SNPs (rs1885301, rs7910642, and rs2804402) not present on the chip were genotyped by direct sequencing at the University of California, San Francisco genetics core facility.

Genetic association analysis. To avoid potential spurious associations as a result of population substructure and admixture, analysis was restricted to 56 Caucasian PAH patients. Selected SNPs were checked for deviation from Hardy–Weinberg equilibrium and filtered for minor allele frequency >5% and linkage disequilibrium <0.8. After filtering, 12 SNPs in five genes—*CYP2C9* (rs1799853 and rs1057910), *SLCO1B1* (rs11045819, rs2306283, and rs4149056), *SLCO1B3* (rs7311358), *ABCB11* (rs2287622), and *ABCC2* (rs717620, rs2273697, rs1885301, rs2804402, and rs7910642)—were analyzed for associations with changes in AST and ALT activity (Outcome 1) and occurrence of DILI (Outcome 2) using linear and logistic regression analyses or Fisher's exact test. An additive inheritance model was applied in the linear regression analysis, and a dominant inheritance model was used for the Fisher's exact test. Linear regression analysis was performed in two steps. First, each clinical covariate and each SNP were tested for associations in univariate analyses, and those with $P < 0.2$ were carried into multivariate analyses. Multivariate models were adjusted for significant clinical covariates with inclusion of SNPs from step 1. Associations with $P < 0.05$ in multivariate analyses were considered significant. A multivariate analysis for genetic associations with DILI was not performed due to the small size of the cohort and the DILI group. Post hoc power calculations for the genetic associations with ALT and AST were conducted for effect sizes equal to the observed effect estimates using the power.t.test function in R v. 3.0.1. Post hoc power for Fisher's exact tests of the association of genetic carrier status with DILI were conducted using the power.fisher.test function from the statmod library in R v. 3.0.1 with 10,000 simulated data sets.

Hepatocyte toxicity assay. The assay was performed as described previously.[49] Briefly, human hepatocytes were plated at 0.3×10^5 cells/well in collagen I-coated plates. Four hours after plating, cells were preincubated for 16 h with 0.5 mmol/l 1-aminobenzotriazole (Sigma-Aldrich, St Louis, MO). Subsequently, medium was replaced with fresh medium containing bosentan, its metabolite Ro 48–5033, or chlorpromazine as a positive control at varying concentrations (0–100 μmol/l) and incubated for an additional 48 h. Dimethyl sulfoxide was used as the negative control. At the end of incubation, cell viability was assessed by measuring adenosine-5'-triphosphate levels using CellTiter-Glo and cellular glutathione levels using GSH-Glo following the manufacturer's protocols (Promega, Madison, WI). Data are presented as mean percentage of control ± SD of triplicate experiments. Half-maximal inhibitory concentration values were calculated using Graph Pad Prism (v. 5).

Enzyme kinetic studies with HLMs and recombinant CYP2C9*1, CYP2C9*2, and CYP2C9*3 enzymes. Bosentan was purchased from Waterstone Technology (Carmel, IN). HLMs and rCYP450 reference enzymes were purchased from BD Biosciences (San Jose, CA). The CYP2C9*1, CYP2C9*2, and CYP2C9*3 enzymes for functional comparison experiments were purchased from Cypex Limited (Dundee, UK). Enzyme activities were measured by parent drug disappearance and metabolite formation in incubations with 0.1 μmol/l, 1 μmol/l, and 10 μmol/l bosentan. All incubations were performed in 100 mmol/l phosphate buffer (pH 7.4). The final protein concentration was 0.5 mg/ml in HLM incubations, and the enzyme concentration was 40 pmol/ml in incubations with recombinant CYP2C9 enzymes. All incubations were performed at 37 °C for 60 min. Reactions were initiated by adding nicotinamide adenine dinucleotide phosphate (final concentration of 10 mmol/l) and were terminated by adding a 3X volume of acetonitrile. Samples were vortexed for 5 min before centrifugation at 2,690*g* for 10 min. The supernatant was transferred to new tubes and dried down at room temperature with a Labconco Centrivap DNA Concentrator. It was then reconstituted with a water:acetonitrile mixture (2:1), followed by vortexing for 5 min and centrifugation at 11,000*g* for 10 min. The supernatant was analyzed by liquid chromatography–tandem mass spectrometry. Negative controls consisting of incubation matrix without substrate were incubated under identical conditions. Data are presented as mean ± SD of triplicate experiments. Statistical analysis was performed using two-way analysis of variance; $P < 0.05$ was considered significant.

Functional assay of bosentan uptake by OATP1B1 reference and variant proteins. Stable cell lines expressing the OATP1B1 reference and variant transporters have been described elsewhere.[50] Uptake assays were performed in triplicate on poly-D-lysine-coated 48-well plates (BD Biosciences, CA). Empty vector, OATP1B1 reference, and the Pro155Thr variant (*rs11045819*, *C463A*)–expressing cells were seeded at 1.5×10^5 cells/well in complete Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum. On the following day, medium was supplemented with 5 mmol/l sodium butyrate, and cells were incubated for another 24 h. Twenty-four hours later, the cells were washed with warm Krebs–Henseleit buffer and incubated with 1, 5, 10, 25, 50, or 100 μmol/l bosentan in Krebs–Henseleit buffer for 5 min at 37 °C. After accumulation, cells were washed twice with ice-cold Krebs–Henseleit buffer and were lysed with a solution of 10% sodium dodecyl sulfate and 1 N NaOH. Bosentan was extracted with two volumes of acetonitrile. An aliquot of the cell lysate was used to determine protein concentration with a bicinchoninic acid protein assay kit (Pierce Biotechnology, IL), and bosentan levels were normalized to total protein concentrations. Data are presented as mean ± SD of triplicate experiments. Statistical analysis was performed using two-way analysis of variance; $P < 0.05$ was considered significant.

Chromatographic and mass spectrometric conditions for measuring bosentan and its metabolites. Liquid chromatography–mass spectrometry analysis was conducted using a Thermo LTQ XL coupled to an ultra-high-pressure liquid chromatography device (Accela Pump), an Accela autosampler, and an Accela photodiode array detector (San Jose, CA). The mass spectrometric conditions were set as follows: positive mode: electrospray ionization, capillary voltage: 16 V, source voltage: 4.5 kV, tube lens voltage: 95 V, capillary temperature: 350 °C, sheath gas flow: 57 units, auxiliary gas flow: 24 units, and sweep gas flow: 5 units, using full-scan, MS^n, and targeted MS^n scan modes. The high-pressure liquid chromatography column used for both systems was a Thermo Hypersil Gold C18 (100 × 2.1 mm, 1.9 μm; Thermo Scientific, Pittsburgh, PA). The solvent system consisted of solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in acetonitrile). Solvent B was delivered initially at 5%, held for 1 min, increased to 95% via a 25-min gradient, then decreased back to 5% at 26 min, and equilibrated for 4 min at a flow rate of 400 μl/min and a total run time of 30 min. The injection volume was 20 μl.

SUPPLEMENTARY MATERIAL is linked to the online version of the paper at http://www.nature.com/cpt

ACKNOWLEDGMENTS
This work was funded by National Institutes of Health grants GM61390 and GM32165, the Foundation for Cardiac Research, and the Affymetrix Early Access Program.

AUTHOR CONTRIBUTIONS

S.M.M. and D.L.K. wrote the manuscript. S.M.M., T.D.M., J.H., A.E.R., C.K., D.M., A.H.B.W., J.B.S., and D.L.K. designed the research. S.M.M., E.A.K., H.L., J.S., and C.Z. performed the research. S.M.M., N.B., J.M., H.L., J.S., C.Z., J.H., W.-C.H., J.S.W., and D.L.K. analyzed the data.

CONFLICT OF INTEREST

The authors declared no conflict of interest.

Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?

✓ Bosentan (Tracleer) is a dual endothelin receptor antagonist prescribed as first-line treatment in PAH patients. DILI (10–12% incidence) is the most serious adverse effect of bosentan.

WHAT QUESTION DID THIS STUDY ADDRESS?

✓ This study was designed to identify genetic markers associated with bosentan-induced liver injury in PAH patients.

WHAT THIS STUDY ADDS TO OUR KNOWLEDGE

✓ CYP2C9*2 was strongly associated with ALT and AST increase and with occurrence of DILI ($\beta = 2.16$, $P = 0.024$; $\beta = 1.92$, $P = 0.016$; and odds ratio 95% confidence interval = 2.29 – infinity, $P = 0.003$, respectively). Bosentan metabolism *in vitro* by CYP2C9*2 is moderately reduced as compared with that by CYP2C9*1 and is comparable to metabolism by CYP2C9*3. *CYP2C9*2* is a potential genetic marker for prediction of bosentan-induced liver injury.

HOW THIS MIGHT CHANGE CLINICAL PHARMACOLOGY AND THERAPEUTICS

✓ This genetic information may be of use in guiding endothelin receptor antagonist therapy in pulmonary hypertension and prevention of bosentan-induced liver injury.

© 2013 American Society for Clinical Pharmacology and Therapeutics

1. McLaughlin, V.V. *et al.*; American College of Cardiology Foundation Task Force on Expert Consensus Documents; American Heart Association; American College of Chest Physicians; American Thoracic Society, Inc; Pulmonary Hypertension Association. ACCF/AHA 2009 expert consensus document on pulmonary hypertension a report of the American College of Cardiology Foundation Task Force on Expert Consensus Documents and the American Heart Association developed in collaboration with the American College of Chest Physicians; American Thoracic Society, Inc.; and the Pulmonary Hypertension Association. *J. Am. Coll. Cardiol*. **53**, 1573–1619 (2009).
2. Tuder, R.M. *et al*. Prostacyclin synthase expression is decreased in lungs from patients with severe pulmonary hypertension. *Am. J. Respir. Crit. Care Med*. **159**, 1925–1932 (1999).
3. Christman, B.W. *et al*. An imbalance between the excretion of thromboxane and prostacyclin metabolites in pulmonary hypertension. *N. Engl. J. Med*. **327**, 70–75 (1992).
4. Stewart, D.J., Levy, R.D., Cernacek, P. & Langleben, D. Increased plasma endothelin-1 in pulmonary hypertension: marker or mediator of disease? *Ann. Intern. Med*. **114**, 464–469 (1991).
5. Giaid, A. & Saleh, D. Reduced expression of endothelial nitric oxide synthase in the lungs of patients with pulmonary hypertension. *N. Engl. J. Med*. **333**, 214–221 (1995).
6. Thenappan, T., Shah, S.J., Rich, S. & Gomberg-Maitland, M. A USA-based registry for pulmonary arterial hypertension: 1982-2006. *Eur. Respir. J*. **30**, 1103–1110 (2007).
7. Dingemanse, J. & van Giersbergen, P.L. Clinical pharmacology of bosentan, a dual endothelin receptor antagonist. *Clin. Pharmacokinet*. **43**, 1089–1115 (2004).
8. Channick, R.N. *et al*. Effects of the dual endothelin-receptor antagonist bosentan in patients with pulmonary hypertension: a randomised placebo-controlled study. *Lancet* **358**, 1119–1123 (2001).
9. Rubin, L.J. *et al*. Bosentan therapy for pulmonary arterial hypertension. *N. Engl. J. Med*. **346**, 896–903 (2002).
10. Denton, C.P. *et al.*; TRacleer Use in PAH associated with Scleroderma and Connective Tissue Diseases (TRUST) Investigators. Long-term effects of bosentan on quality of life, survival, safety and tolerability in pulmonary arterial hypertension related to connective tissue diseases. *Ann. Rheum. Dis*. **67**, 1222–1228 (2008).
11. Treiber, A., Schneiter, R., Häusler, S. & Stieger, B. Bosentan is a substrate of human OATP1B1 and OATP1B3: inhibition of hepatic uptake as the common mechanism of its interactions with cyclosporin A, rifampicin, and sildenafil. *Drug Metab. Dispos*. **35**, 1400–1407 (2007).
12. Burgess, G., Hoogkamer, H., Collings, L. & Dingemanse, J. Mutual pharmacokinetic interactions between steady-state bosentan and sildenafil. *Eur. J. Clin. Pharmacol*. **64**, 43–50 (2008).
13. van Giersbergen, P.L., Halabi, A. & Dingemanse, J. Single- and multiple-dose pharmacokinetics of bosentan and its interaction with ketoconazole. *Br. J. Clin. Pharmacol*. **53**, 589–595 (2002).
14. Dingemanse, J., van Giersbergen, P.L., Patat, A. & Nilsson, P.N. Mutual pharmacokinetic interactions between bosentan and lopinavir/ritonavir in healthy participants. *Antivir. Ther. (Lond.)* **15**, 157–163 (2010).
15. Fouassier, L. *et al*. Contribution of mrp2 in alterations of canalicular bile formation by the endothelin antagonist bosentan. *J. Hepatol*. **37**, 184–191 (2002).
16. Fattinger, K. *et al*. The endothelin antagonist bosentan inhibits the canalicular bile salt export pump: a potential mechanism for hepatic adverse reactions. *Clin. Pharmacol. Ther*. **69**, 223–231 (2001).
17. Mano, Y., Usui, T. & Kamimura, H. Effects of bosentan, an endothelin receptor antagonist, on bile salt export pump and multidrug resistance-associated protein 2. *Biopharm. Drug Dispos*. **28**, 13–18 (2007).
18. Hartman, J.C., Brouwer, K., Mandagere, A., Melvin, L. & Gorczynski, R. Evaluation of the endothelin receptor antagonists ambrisentan, darusentan, bosentan, and sitaxsentan as substrates and inhibitors of hepatobiliary transporters in sandwich-cultured human hepatocytes. *Can. J. Physiol. Pharmacol*. **88**, 682–691 (2010).
19. Stieger, B., Meier, Y. & Meier, P.J. The bile salt export pump. *Pflugers Arch*. **453**, 611–620 (2007).
20. Ho, R.H. *et al*. Polymorphic variants in the human bile salt export pump (BSEP; ABCB11): functional characterization and interindividual variability. *Pharmacogenet. Genomics* **20**, 45–57 (2010).
21. Lang, C. *et al*. Mutations and polymorphisms in the bile salt export pump and the multidrug resistance protein 3 associated with drug-induced liver injury. *Pharmacogenet. Genomics* **17**, 47–60 (2007).
22. Pauli-Magnus, C. *et al*. Sequence analysis of bile salt export pump (ABCB11) and multidrug resistance p-glycoprotein 3 (ABCB4, MDR3) in patients with intrahepatic cholestasis of pregnancy. *Pharmacogenetics* **14**, 91–102 (2004).
23. Dixon, P.H. *et al*. Contribution of variant alleles of ABCB11 to susceptibility to intrahepatic cholestasis of pregnancy. *Gut* **58**, 537–544 (2009).
24. Humbert, M., Segal, E.S., Kiely, D.G., Carlsen, J., Schwierin, B. & Hoeper, M.M. Results of European post-marketing surveillance of bosentan in pulmonary hypertension. *Eur. Respir. J*. **30**, 338–344 (2007).
25. Daly, A.K., Aithal, G.P., Leathart, J.B., Swainsbury, R.A., Dang, T.S. & Day, C.P. Genetic susceptibility to diclofenac-induced hepatotoxicity: contribution of UGT2B7, CYP2C8, and ABCC2 genotypes. *Gastroenterology* **132**, 272–281 (2007).
26. Choi, J.H. *et al*. MRP2 haplotypes confer differential susceptibility to toxic liver injury. *Pharmacogenet. Genomics* **17**, 403–415 (2007).
27. Nguyen, T.D. *et al*. Functional characterization of ABCC2 promoter polymorphisms and allele-specific expression. *Pharmacogenomics J*. (2012).
28. Hirouchi, M. *et al*. Characterization of the cellular localization, expression level, and function of SNP variants of MRP2/ABCC2. *Pharm. Res*. **21**, 742–748 (2004).
29. Haenisch, S., May, K., Wegner, D., Caliebe, A., Cascorbi, I. & Siegmund, W. Influence of genetic polymorphisms on intestinal expression and rifampicin-type induction of ABCC2 and on bioavailability of talinolol. *Pharmacogenet. Genomics* **18**, 357–365 (2008).

30. Venitz, J., Zack, J., Gillies, H., Allard, M., Regnault, J. & Dufton, C. Clinical pharmacokinetics and drug-drug interactions of endothelin receptor antagonists in pulmonary arterial hypertension. *J. Clin. Pharmacol*. **52**, 1784–1805 (2012).
31. Lee, C.R., Goldstein, J.A. & Pieper, J.A. Cytochrome P450 2C9 polymorphisms: a comprehensive review of the in-vitro and human data. *Pharmacogenetics* **12**, 251–263 (2002).
32. Cavallari, L.H., Shin, J. & Perera, M.A. Role of pharmacogenomics in the management of traditional and novel oral anticoagulants. *Pharmacotherapy* **31**, 1192–1207 (2011).
33. van der Weide, J., Steijns, L.S., van Weelden, M.J. & de Haan, K. The effect of genetic polymorphism of cytochrome P450 CYP2C9 on phenytoin dose requirement. *Pharmacogenetics* **11**, 287–291 (2001).
34. Tang, C. *et al*. In-vitro metabolism of celecoxib, a cyclooxygenase-2 inhibitor, by allelic variant forms of human liver microsomal cytochrome P450 2C9: correlation with CYP2C9 genotype and in-vivo pharmacokinetics. *Pharmacogenetics* **11**, 223–235 (2001).
35. Lee, C.R., Pieper, J.A., Frye, R.F., Hinderliter, A.L., Blaisdell, J.A. & Goldstein, J.A. Tolbutamide, flurbiprofen, and losartan as probes of CYP2C9 activity in humans. *J. Clin. Pharmacol*. **43**, 84–91 (2003).
36. Kirchheiner, J. & Brockmöller, J. Clinical consequences of cytochrome P450 2C9 polymorphisms. *Clin. Pharmacol. Ther*. **77**, 1–16 (2005).
37. Chaudhry, A.S. *et al*. CYP2C9*1B promoter polymorphisms, in linkage with CYP2C19*2, affect phenytoin autoinduction of clearance and maintenance dose. *J. Pharmacol. Exp. Ther*. **332**, 599–611 (2010).
38. Weiss, J., Theile, D., Rüppell, M.A., Speck, T., Spalwisz, A. & Haefeli, W.E. Interaction profile of macitentan, a new non-selective endothelin-1 receptor antagonist, *in vitro*. *Eur. J. Pharmacol*. **701**, 168–175 (2013).
39. Belic, A. *et al*. Investigation of the CYP2C9 induction profile in human hepatocytes by combining experimental and modelling approaches. *Curr. Drug Metab*. **10**, 1066–1074 (2009).
40. Cooper, G.M. *et al*. A genome-wide scan for common genetic variants with a large influence on warfarin maintenance dose. *Blood* **112**, 1022–1027 (2008).
41. Teichert, M. *et al*. A genome-wide association study of acenocoumarol maintenance dosage. *Hum. Mol. Genet*. **18**, 3758–3768 (2009).
42. Niemi, M., Pasanen, M.K. & Neuvonen, P.J. Organic anion transporting polypeptide 1B1: a genetically polymorphic transporter of major importance for hepatic drug uptake. *Pharmacol. Rev*. **63**, 157–181 (2011).
43. Srivastava, A., Srivastava, A., Srivastava, N., Choudhuri, G. & Mittal, B. Organic anion transporter 1B1 (SLCO1B1) polymorphism and gallstone formation: High incidence of Exon4 CA genotype in female patients in North India. *Hepatol. Res*. **41**, 71–78 (2011).
44. Couvert, P., Chapman, M.J. & Carrié, A. Impact of genetic variation in the SLCO1B1 gene on statin efficacy in low-density lipoprotein cholesterol-lowering therapy. *Pharmacogenomics* **12**, 137–139 (2011).
45. Geng, F. *et al*. The association of the UGT1A8, SLCO1B3 and ABCC2/ABCG2 genetic polymorphisms with the pharmacokinetics of mycophenolic acid and its phenolic glucuronide metabolite in Chinese individuals. *Clin. Chim. Acta* **413**, 683–690 (2012).
46. Schwarz, U.I. *et al*. Identification of novel functional organic anion-transporting polypeptide 1B3 polymorphisms and assessment of substrate specificity. *Pharmacogenet. Genomics* **21**, 103–114 (2011).
47. Benichou, C., Danan, G. & Flahault, A. Causality assessment of adverse reactions to drugs–II. An original model for validation of drug causality assessment methods: case reports with positive rechallenge. *J. Clin. Epidemiol*. **46**, 1331–1336 (1993).
48. Danan, G. & Benichou, C. Causality assessment of adverse reactions to drugs–I. A novel method based on the conclusions of international consensus meetings: application to drug-induced liver injuries. *J. Clin. Epidemiol*. **46**, 1323–1330 (1993).
49. Pai, R. *et al*. Antibody-mediated inhibition of fibroblast growth factor 19 results in increased bile acids synthesis and ileal malabsorption of bile acids in cynomolgus monkeys. *Toxicol. Sci*. **126**, 446–456 (2012).
50. Tamraz, B. *et al*. OATP1B1-related drug-drug and drug-gene interactions as potential risk factors for cerivastatin-induced rhabdomyolysis. *Pharmacogenet. Genomics* **23**, 355–364 (2013).