Multiple Compound-Related Adverse Properties Contribute to Liver Injury Caused by Endothelin Receptor Antagonists

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ABSTRACT

Drug-induced liver injury has been observed in patients treated with the endothelin receptor antagonists sitaxentan and bosentan, but not following treatment with ambrisentan. The aim of our studies was to assess the possible role of multiple contributory mechanisms in this clinically relevant toxicity. Inhibition of the bile salt export pump (BSEP) and multidrug resistance-associated protein 2 was quantified using membrane vesicle assays. Inhibition of mitochondrial respiration in human liver–derived HuH-7 cells was determined using a Seahorse analyzer. Cytochrome P450 (P450)–independent and P450-mediated cell toxicity was assessed using transfected SV40-T-antigen–immortalized human liver epithelial (THLE) cell lines. Exposure-adjusted assay ratios were calculated by dividing the maximum human drug plasma concentrations by the IC50 or EC50 values obtained in vitro. Covalent binding (CVB) of radiolabeled drugs to human hepatocytes was quantitated, and CVB body burdens were calculated by adjusting CVB values for fractional drug turnover in vitro and daily therapeutic dose. Sitaxentan exhibited positive exposure-adjusted signals in all five in vitro assays and a high CVB body burden. Bosentan exhibited a positive exposure-adjusted signal in one assay (BSEP inhibition) and a moderate CVB body burden. Ambrisentan exhibited no positive exposure-adjusted assay signals and a low CVB body burden. These data indicate that multiple mechanisms contribute to the rare, but potentially severe liver injury caused by sitaxentan in humans; provide a plausible rationale for the markedly lower propensity of bosentan to cause liver injury; and highlight the relative safety of ambrisentan.

Introduction

Numerous different drugs may cause liver injury in humans, which occurs infrequently in certain susceptible patients (i.e., is idiosyncratic), and is not evident from safety studies undertaken in animals. The consequences of drug-induced liver injury (DILI) range from mild and asymptomatic liver injury, which results in elevated plasma levels of alanine aminotransferase (ALT) and other enzymes released from damaged liver cells, to clinically concerning liver dysfunction that, in the most severe instances, may result in acute liver failure (Yuan and Kaplowitz, 2013). Consequently, DILI is a leading cause of withdrawal of licensed drugs, failed drug registration, and human ill health (Lasser et al., 2002; Yuan and Kaplowitz, 2013). The underlying mechanisms are complex and include both drug-related adverse properties, which explain why some drugs cause DILI, whereas others do not, and patient-related factors that influence individual susceptibility (Thompson et al., 2011; Yuan and Kaplowitz, 2013). Drug-related adverse properties that have been implicated in the development of DILI include covalent and noncovalent modification of cellular macromolecules caused by electrophilic reactive metabolites (Park et al., 2011), intrinsic cell cytotoxicity (Gustafsson et al., 2014), impaired mitochondrial function (Nadanaciva and Will, 2011), inhibition of hepatocyte bile salt export pump (BSEP) activity (Dawson et al., 2012), stimulation of innate and adaptive immune responses (Yuan and Kaplowitz, 2013), and relatively high in vivo drug dose and exposure (Lammert et al., 2008). Patient-related susceptibility is complex and includes...
both genetically determined and nongenetic factors (Yuan and Kaplowitz, 2013).

Endothelin receptor antagonists (ERAs) are an important class of drugs in which compounds have been found to cause DILI in humans. ERAs are indicated for the treatment of pulmonary arterial hypertension, to improve exercise capacity and delay clinical worsening. Cases of infrequent, but severe hepaticitis-like drug reactions, which were not evident at the time of registration and included several instances of acute liver failure, were observed postmarketing in patients treated with sitaxentan, which led to the withdrawal of the drug in 2010 (Galié et al., 2011). Bosentan has been given a cautionary DILI “black box” warning by the Food and Drug Administration (FDA), and monthly monitoring of liver function is required, as a 10% incidence of serum aminotransferase elevations and several cases of acute and severe liver injury have been observed in treated patients (Humbert et al., 2007; Eriksson et al., 2011). Furthermore, an increased frequency of serum ALT elevations in patients treated with high doses of bosentan was observed during its clinical evaluation (Fattinger et al., 2001). However, no cases of liver failure caused by bosentan have been reported. In contrast, ambrisentan was found not to cause concerning plasma ALT elevations (>3-fold upper limit of the normal reference range) during clinical trials, nor to cause DILI postlicensing (even in cohorts of patients who previously had discontinued bosentan or sitaxentan due to plasma ALT elevations) (McGoone et al., 2009; Frampton, 2011). Consequently, although ambrisentan initially was issued a DILI black box warning by the FDA, this was subsequently removed (http://www.fda.gov/Drugs/DrugSafety/ucm245852).

The mechanisms that underlie the different DILI propensities of sitaxentan, bosentan, and ambrisentan in humans remain poorly defined, and sitaxentan has been reported not to cause DILI in animal species (Owen et al., 2012). One important contributory process is considered to be BSEP inhibition, which has been described for bosentan and sitaxentan (Fattinger et al., 2001; Lepist et al., 2014). However, the role played by other possible drug-related adverse properties has not been as well explored. In the present studies, we have evaluated these drugs in vitro in a multiparametric assay panel, which was based on that described by Thompson et al. (2012) and included assessment of biliary transporter inhibition, impairment of mitochondrial function, toxicity to cell lines expressing either no human cytochrome P450 (P450) enzymes or individual human P450 enzymes, and metabolic bioactivation in human hepatocytes. To aid interpretation of the potential in vivo significance of these in vitro data, exposure-adjusted assay ratios were calculated by dividing the reported steady state maximum human plasma drug concentrations (C_{max,ss}) by the in vitro IC_{50} or EC_{50} values.

Materials and Methods

Compound structures, including the positions of the radiolabels, are shown in Fig. 1. Bosentan (Ro 47-9020; 4-tert-butyl-N-[4-(2-hydroxy-ethoxy)-5-(2-methoxy-phenoxy)-2,2′-bipyrimidin-4-yl]-benzenesulfonamide) (Clozel et al., 1994) was synthesized by Medicinal Chemistry, AstraZeneca (Molndal, Sweden). Sitaxentan (TBC11251; N-[4-chloro-3-methyl-1,2-oxazol-5-yl]-2-[2-46-methyl-2H,1,3-benzodioxol-5-yl]acetilthiophene-3-sulfonamide) (Wu et al., 1997) and ambrisentan (BSF208075; LU208075; [2S]-2-[4-(6-dimethylpyrimidin-2-yl)oxyl]-3-methoxy-3,3-diphenylpropanoic acid) (Riechers et al., 1996) were obtained from Chemtrionica AB (Stockholm, Sweden). THLE cell lines (1A2, 2C9, 2C19, 2D6, 3A4, and Null) were obtained from Nestec (Lausanne, Switzerland). HuH-7 cells were obtained from the RIKEN BioResource Center (Ibaraki, Japan) and were verified by genotyping. Cryopreserved pooled human hepatocytes (10 donors, female and male) were from BioreclamationIVT (Baltimore, MD). Baculovirus expressing human BSEP (ABC111) was provided by B. Stiegler (University of Zurich, Zurich, Switzerland). CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay (MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium]) was obtained from Promega UK (Southampton, UK). BioCoat Collagen-I Cellware 96-well plates were obtained from BD Biosciences (Bedford, UK). [3H]Taurocholic acid (1 mCi/ml) was purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA). CDF [56-carboxy-2′, 7′-dichlorofluorescein], Dulbecco’s modified Eagle’s media (DMEM), unbuffered DMEM, carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (PCPP), retorone, oligomycin, antimycin A, and all other reagents were obtained from Sigma-Aldrich (Poole, UK). Basal Pasadena Foundation for Medical Research Medium (PMFR)-4 medium was obtained from Invitrogen (Paisley, UK). XFe96 sensor cartridges, XFe96-well plates, calibration buffer, and calibration plates were purchased from Seahorse Biosciences (Billerica, MA).

Synthesis of Radiolabeled ERAs. Tritium labeling of [7-3H]sitaxentan (N-[4-chloro-3-methyl-1,2-oxazol-5-yl]-2-[2-7-3H]-6-methyl-2H,1,3-benzodioxol-5-yl]acetilthiophene-3-sulfonamide), [4-3H]sitaxentan (N-[4-chloro-3-methyl-1,2-oxazol-5-yl]-2-[2-4-3H]-6-methyl-2H,1,3-benzodioxol-5-yl]acetilthiophene-3-sulfonamide), [3H]bosentan (4-tert-butyl-N-[6-(2-hydroxy-ethoxy)-5-(5-[3H]2-methoxy-phenoxy)-2,2′-bipyrimidin-4-yl]-benzenesulfonamide), and [3H]ambrisentan ([2S]-2-[4-(6-dimethylpyrimidin-2-yl)oxyl]-3-methoxy-3-phenyl-[4-3H]phenylpropionic acid) (see Fig. 1) was undertaken in two steps based on that described by Thompson et al. (2012) and included assessment of biliary transporter inhibition, impairment of mitochondrial function, toxicity to cell lines expressing either no human cytochrome P450 (P450) enzymes or individual human P450 enzymes, and metabolic bioactivation in human hepatocytes. To aid interpretation of the potential in vivo significance of these in vitro data, exposure-adjusted assay ratios were calculated by dividing the reported steady state maximum human plasma drug concentrations (C_{max,ss}) by the in vitro IC_{50} or EC_{50} values.
spectroscopy. Halogenation of bosentan was effected using 1.5 equivalents of N-iodosuccinimide in neat trifluoroacetic acid, and the resulting iodide was purified by preparative HPLC. Halogenation of ambrisentan was effected using an excess of Br₂ in acetic acid, and the resulting mixture of diastereomeric bromides was purified by preparative HPLC. There was no evidence of diastereomeric separation during the purification. Tritiodehalogenation of all four compounds were purified by preparative HPLC. The radiochemical purities of [7-3H]sitaxentan, [4-3H]sitaxentan, [4H]bosentan, and [4H]ambrisentan were determined by HPLC to be 91%, 98%, 99%, and 99%, respectively, and the specific activities were determined by liquid chromatography–mass spectrometry to be 270, 700, 980, and 950 kBq/nmol, respectively. Both [7-3H]sitaxentan and [4-3H]sitaxentan are very sensitive to handling and particularly during isolation. Once isolated in a solution of EtOH, the radiochemical purity of both compounds declined rapidly. To minimize the radiochemical decomposition between isolation and covalent binding assay, each compound was repurified the day prior to use, and a stock solution of the compound in dimethylsulfoxide (DMSO) was prepared to limit the need for further handling. Radiochemical purity determination immediately prior to use showed the purity of [7-3H]sitaxentan to be 89% and [4-3H]sitaxentan to be 88%.

**BSEP and Multidrug Resistance-Associated Protein 2 Transporter Activity.** The effects of the ERA compounds on ATP-dependent uptake of probe substrates into inside-out membrane vesicles prepared from baculovirus-infected Spodoptera frugiperda Sf21 insect cells expressing human BSEP or multidrug resistance-associated protein 2 (MRP2) proteins were quantified using rapid filtration assays in 96-well plate format, as described by Rodrigues et al. (2013). The BSEP substrate was [3H]taurocholate (PerkinElmer, Waltham, MA), and the MRP2 substrate was CDF. The final DMSO concentration in all incubations was 2% (v/v). For the studies in MRP2 vesicles, incubations were also undertaken using 5 mM AMP in place of ATP at each test compound concentration to quantify substrate accumulation independent of active transport.

**Evaluation of Mitochondrial Respiratory Function.** Oxygen consumption rate (OCR), maximum respiration rate (MRR), and extracellular acidification rate (ECAR) were determined in HuH-7 cells using an XF20i-flux analyzer (Seahorse Biosciences), as described by Brand and Nicholls (2011). The cells were exposed to the ERA compounds, following which mitochondrial stress was evaluated by subsequent consecutive addition of oligomycin, FCCP, and rotenone plus antimycin A (Rot/AA), according to the manufacturer’s guidelines.

Cells that had been cultured at 37°C in tissue culture flasks in DMEM (D6429) supplemented with 10% fetal bovine serum (FBS), in 5% CO₂, 95% air, were seeded at 20,000 cells/well onto XFe96 plates (Seahorse Biosciences) and left overnight to attach. The culture medium was replaced with unbuffered DMEM assay medium (D5030) supplemented with 10 mM glucose, 1 mM pyruvate, 2 mM l-alanyl-glutamine, and 0.1% fatty acid free bovine serum albumin (medium pH 7.4, 37°C) immediately prior to each assay, and cells were then incubated in a CO₂-free incubator at 37°C for 90 minutes. Basal OCRs were determined on three sequential readings, each comprising a 3-minute mix and 4-minute read cycle. Concentrated solutions (10 ×) of the ERA compounds or DMSO vehicle alone in assay medium were then injected (injection 1). The final DMSO concentration in all incubations was 0.5% (v/v). After injection 1, OCR was measured for an additional six cycles and also following further addition of oligomycin (1 μM; injection 2), FCCP (1.5 μM; injection 3), and Rot/AA (each at 1 μM; injection 4). For OCR concentration time profiles, values were normalized to the last reading before ERA or vehicle addition (injection 1). To calculate EC50 values, nonmitochondrial OCR values after administration of Rot/AA (last reading) were subtracted from all other readings, and values were expressed relative to the corresponding DMSO control value of the last reading before oligomycin addition (injection 2) for OCR and the last reading before Rot/AA addition (injection 4) for MRR, respectively.

**Evaluation of Toxicity to THLE Cell Lines.** THLE-1A2, 2C9, 2C19, 2D6, 3A4, or THLE-Null cell lines were cultured in PMFR4™ medium (basal PMFR4 medium plus 2 mM GlutaMAX, 10 μg/ml recombinant human insulin, 5.5 μg/ml human transferrin, 6.7 ng/ml sodium selenite, 1 μM hydrocortisone, 0.5 ng/ml epidermal growth factor, 35 μg/ml bovine pituitary extract, 0.35 mM retinoic acid, 3% FBS, 150 μg/ml G418), in BioCoat Collagen-I 96-well plates, and were exposed for 18 hours to the γ-glutamylcysteine synthetase inhibitor buthionine sulfoximine (BSO; 80 μM) in PMFR4™ medium (basal PMFR™ medium without insulin, transferrin, sodium selenite, or FBS), or to PMFR4™ medium alone, as described previously (Foster et al., 2013). Cells were then incubated with the ERA compounds, or DMSO vehicle alone (0.2% (v/v)), in the presence or absence of BSO for an additional 24 hours. The percentage of viable cells relative to the vehicle-treated control wells was determined for each well using the CellTiter-96 AQueous Non-Radioactive Cell Proliferation Assay (MTS; Promega UK).

**Covalent Binding Studies in Human Hepatocytes.** Studies were undertaken in pooled cryopreserved human hepatocytes using the semi-automated covalent binding (CVB) assay reported previously (Thompson et al., 2012) with minor modifications. Stock solutions of [3H]-labeled ambrisentan and bosentan (10 mM) were prepared by evaporating the ethanol from the labeled compound and dissolving it in DMSO containing unlabeled compound (10 mM). Due to poor isotope stability, stock solutions of [4H]- and [7H]-labeled sitaxentan (500 μM) were prepared directly in DMSO that contained unlabeled compound, acetonitrile, and water. Hepatocyte suspensions (585 μl, 1,000,000 cells/ml) were preincubated for 5 minutes at 37°C; incubations were initiated by the addition of preheated radiolabeled compounds (65 μl) and were performed for 4 hours. The final DMSO and acetonitrile concentrations were 0.1% and 1%, respectively, and all incubations were carried out in triplicate. After addition of test compound (10 μM final concentrations for all compounds) and at the end of the incubation (i.e., at 0 and 4 hours, respectively), aliquots were taken for CVB and turnover determination (200 and 50 μl, respectively). To account for nonspecific binding, the net covalent binding of each compound was determined by subtracting its 0-hour value from the paired result after the 4-hour incubation. Samples for parent compound turnover determination were analyzed on an Agilent 1100 series HPLC system (Agilent Technologies, Santa Clara, CA) coupled to a Waters Quattro Ultima (Waters, Milford, MA). A Sunniest RP-AQUA series HPLC system (Agilent Technologies, Santa Clara, CA) coupled to an Agilent 1100 series HPLC system (Agilent Technologies, Santa Clara, CA) coupled to a Waters Quattro Ultima (Waters, Milford, MA). A Sunniest RP-AQUA series HPLC system (Agilent Technologies, Santa Clara, CA) coupled to an Agilent 1100 series HPLC system (Agilent Technologies, Santa Clara, CA) coupled to a Waters Quattro Ultima (Waters, Milford, MA), and the MRP2 substrate was CDF. The final DMSO concentration in all incubations was 2% (v/v). For the studies in MRP2 vesicles, incubations were also undertaken using 5 mM AMP in place of ATP at each test compound concentration to quantify substrate accumulation independent of active transport.
Statistical Analyses. Data are expressed as means ± S.E.M. IC₅₀ and EC₅₀ values were determined by nonlinear regression using GraphPad Prism (GraphPad Software, La Jolla, CA) and are expressed as geometric means ± 95% confidence interval. Statistical analyses were performed in GraphPad Prism by one-tailed analysis of variance.

Results

Effects of ERAs on BSEP and MRP2 Activities. Each of the ERAs caused concentration-dependent inhibition of [³H]taurocholate uptake by membrane vesicles that expressed human BSEP. Sitaxentan was the most potent and ambrisentan the least potent BSEP inhibitor (Fig. 2A; Supplemental Table 1; Table 1). Sitaxentan and bosentan also caused concentration-dependent inhibition of CDF uptake by membrane vesicles that expressed MRP2, and sitaxentan was again the more potent inhibitor (Fig. 2B; Table 1). In contrast, exposure of vesicles expressing MRP2 to ambrisentan caused concentration-dependent stimulation of CDF accumulation, which was energy-dependent (i.e., was not observed when ATP was replaced with AMP) and reached a maximum of 1.5-fold of the vehicle control value at 500 μM (Fig. 2B; Supplemental Fig. 1; Supplemental Table 1).

Effects on Cellular Mitochondrial Function. The influence of ERAs on basal mitochondrial respiration was determined by quantifying their direct effects on OCR and ECAR in human liver-derived HuH-7 cells, which have been shown by previous investigators to be suitable for used in the XFe⁹⁶-flux analyzer (Brand and Nicholls, 2011). The cells were exposed to the drugs and then subsequently to the ATP synthase inhibitor oligomycin, the uncoupler FCCP, and the electron transport chain inhibitors Rot/AA. Cells exposed to DMSO vehicle (injection 1, Fig. 3), that is, cells with unimpaired mitochondrial respiration, exhibited a marked and prompt reduction in basal OCR following the addition of oligomycin (injection 2), which was stimulated above baseline values following subsequent administration of FCCP (injection 3) and then was fully inhibited following addition of Rot/AA (injection 4). The difference between uncoupled cellular OCR following the addition of FCCP and OCR when the electron transport chain is inhibited (by treatment with Rot/AA) is termed MRR (Brand and Nicholls, 2011).

Exposure of HuH-7 cells to 250 μM sitaxentan caused a progressive and marked decrease in basal OCR, which was not further affected by addition of oligomycin, but was increased following the subsequent addition of FCCP (Fig. 3A). This pattern of results is indicative of marked inhibition of MRR by sitaxentan. Less potent and concentration-dependent impairment of MRR was observed when cells were exposed to lower sitaxentan concentrations (8.8–125 μM), which also caused a small extent of basal OCR inhibition (Figs. 3A and 4; Supplemental Table 1; Table 1). Cell exposure to 250 μM bosentan caused a progressive reduction in basal OCR, which was less pronounced than the effect caused by an equivalent concentration of sitaxentan and was unaffected by the subsequent addition of oligomycin or FCCP (Fig. 3B; Table 1). However, no effects on either OCR or MRR were observed at lower bosentan concentrations (Figs. 3B and 4; Table 1). Modest and concentration-dependent inhibition of basal OCR and MRR was observed when cells were exposed to ambrisentan at concentrations up to 125 μM (Figs. 3C and 4; Table 1). This was the maximum concentration of the compound that could be achieved in the assay medium used in the XFe⁹⁶-flux analyzer studies. Cell exposure to the three ERAs also caused marked and concentration-dependent stimulation of ECAR, which provided additional evidence that impaired mitochondrial respiration had occurred (see Supplemental Fig. 2). The effects on ECAR were not quantified because this is primarily a consequence of lactate release following glycolysis and the assay was not optimized for assessment of this metabolic pathway, due to the presence of pyruvate in the cell culture medium.

Toxicity to THLE Cell Lines. The influence of P450 expression on cell toxicity caused by the ERAs was assessed using a panel of human liver–derived THLE cell lines, which expressed either no detectable P450 activities (THLE-Null cells) or the major human hepatic P450 enzymes CYP1A2, CYP2C9, CYP2C19, CYP3A4, or CYP2D6. The value of these cell lines for investigation of P450-independent and P450 metabolite-mediated cell toxicity has been demonstrated previously (Foster et al., 2013; Gustafsson et al., 2014). A marked and concentration-dependent reduction in MTS activity, which is indicative of reduced cell viability (Gustafsson et al., 2014), was observed when THLE-Null cells were exposed to sitaxentan for 24 hours (Fig. 5A; Supplemental Table 1; Table 1). In addition, the toxicity of sitaxentan was potentiated markedly in THLE-3A4 cells, which selectively express CYP3A4. This resulted in a THLE-Null/THLE-3A4 EC₅₀ ratio of 1.8. Bosentan
caused concentration-dependent effects on cell viability when
tested at concentrations that exceeded 100 μM, and under these
conditions there was also a small increase in toxicity to THLE-
3A4 cells, compared with THLE-Null cells (Fig. 5B; Table 1).
Cell toxicity was not observed when either THLE-Null or
THLE-3A4 cells were exposed to equivalent concentrations of
ambrisentan (Fig. 5C; Table 1). The effects of all three ERAs on
MTS activities in THLE cell lines that expressed alternative
human hepatic P450 enzymes (CYP1A2, CYP2C9, CYP2C19,
CYP2D6) were similar to those observed in THLE-Null cells
(Supplemental Fig. 3; Supplemental Table 2). The potencies of
toxicity of the ERAs to THLE cell lines were unaffected by cell
exposure to BSO (Supplemental Figs. 4–6; Supplemental
Table 2). BSO is a potent inhibitor of γ-glutamylcysteine syn-
thetase, which is a key enzyme required to support glutathione
(GSH) synthesis (Griffith and Meister, 1979) and depleted
cellular reduced GSH concentrations by about 85%, to <5 μM
(data not shown).

CVB of Radiolabeled ERAs to Human Hepatocyte
Proteins. CVB studies with sitaxentan were performed using
two tritium-labeled analogs, [4-3H]sitaxentan and [7-3H]sitaxentan; the chemical structures of these and the other
radiolabeled ERAs are shown in Fig. 1. Both sitaxentan
anologs yielded similar levels of CVB to human hepatocyte
proteins, which were markedly higher than the CVB levels
observed with bosentan or ambrisentan (Table 2). No triitated
water was seen with any of the compounds. To adjust the CVB
data for the variable extents of in vitro turnover of the drugs,
\[ f_{CVB} \] values were calculated. The average \[ f_{CVB} \] value for the
tritium-labeled analogs of sitaxentan was 10- and 18-fold
higher than that for bosentan and ambrisentan, respectively.
CVB body burdens were then calculated by multiplying \[ f_{CVB} \]
by the maximum recommended daily therapeutic doses of the
drugs, which ranged from 10 mg q.d. (ambrisentan) to 125 mg
b.i.d. (bosentan; see Table 2). The estimated CVB body burden of
sitaxentan was 4- and 200-fold higher than that of bosentan
and ambrisentan, respectively (Table 2). For sitaxentan, this
CVB body burden was considerably above the threshold of
safety concern (≥1 mg/day) defined in our previous study
(Thompson et al., 2012). Ambrisentan and bosentan, in con-
trast, exhibited low \[ f_{CVB} \] values, and at least for ambrisentan, a
CVB body burden below the threshold of concern. For bosentan,
however, the CVB body burden was above the threshold of
concern due to its relatively high maximum daily dose of
125 mg b.i.d. (Table 2).

Zone Visualization of Aggregated In Vitro Safety
Data and CVB Body Burdens. Initially, the in vitro assay
data were aggregated by assigning each endpoint a binary
score of 0 or 1, depending upon whether the observed potency of
effect was below or above defined threshold values of concern
and then summing these scores (Thompson et al., 2012). The
threshold values were derived by analysis of a substantial
number of drugs reported to cause human DILI and drugs not
reported to cause DILI, as has been described in previous
publications. The threshold of concern for BSEP inhibition
(≥300 μM) was exhibited by 28 of 64 (44%) drugs that caused
human DILI and by 5 of 21 (24%) drugs that did not cause DILI
(Dawson et al., 2012). The threshold value for MRP2 inhibition
(≥300 μM) was exhibited by 8 of 30 drugs that caused human
DILI (27%), and by 0 of 6 drugs (0%) that did not cause DILI
(Thompson et al., 2012). The threshold of concern for THLE-
Null cell toxicity (<200 μM) was exhibited by 21 of 82 drugs
that caused human DILI (26%) and by 1 of 21 drugs (5%) that
did not cause DILI (Gustafsson et al., 2014). The threshold for
potentiated THLE-3A4 cell toxicity (THLE-Null/THLE-3A4
EC50 ratio >1.4) was exhibited by 10 of 82 drugs that caused
DILI (12%) and by 1 of 21 drugs (5%) that did not (Gustafsson
et al., 2014).

Because comparative reference mitochondrial respiration
assay data obtained with toxic and nontoxic drugs have not
been reported, these results were interpreted using a more
empirical approach. All three compounds were assigned
scores of 0 for OCR inhibition, as marked inhibition was only
evident at the highest tested concentration of 250 μM for
sitaxentan and bosentan, and ambrisentan did not markedly
affect OCR. In view of its relatively potent inhibition of MRR
(EC50 = 61 μM), sitaxentan was assigned a score of 1 for this
endpoint, whereas bosentan and ambrisentan exhibited con-
siderably less potent MRR inhibition and were thus assigned
scores of 0.

The binary endpoint scores assigned to each ERA against
each safety assay are summarized in Table 1, as are the
resulting aggregated safety scores. A plot of the aggregated
safety scores against CVB body burden is shown in Fig. 6A.
The plot has been divided into four zones, as proposed by
Thompson et al. (2012) following analysis of 36 drugs. Both
sitaxentan and bosentan exhibited multiple in vitro safety
scores (aggregate score >1) and CVB body burdens ≥1 mg/day
and were assigned to zone 4, although the values obtained
for sitaxentan were markedly greater than for bosentan.
Ambrisentan exhibited a single safety score (BSEP inhibition) and a CVB body burden <1 mg/day, and therefore was assigned to zone 1.

In addition, the impact on the binary safety assay scores of taking account of the human in vivo drug exposure was explored by estimating the ratios between the reported total steady state plasma concentrations ($C_{\text{max,ss}}$) of the drugs (Dingemanse and van Giersbergen, 2004; Scott, 2007; Croxtall and Keam, 2008) at their maximum prescribed doses and the observed in vitro $IC_{50}$ or $EC_{50}$ values. Following the FDA draft guidance on drug interaction studies (http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm292362.pdf), a 10-fold safety margin was used and ratios $\geq 0.1$ were assigned scores of 1, whereas ratios $< 0.1$ were assigned scores of 0. Calculation of the ratios between $C_{\text{max,ss}}$ and THLE-Null/THLE-3A4 $EC_{50}$ ratios was considered inappropriate for estimation of exposure-adjusted potentiated THLE-3A4 cell toxicity and was not undertaken. Instead, $C_{\text{max,ss}}$/THLE-3A4 $EC_{50}$ ratios were estimated. The various calculations and values are summarized in Table 3, and a plot of the resulting exposure-adjusted aggregated safety signals against CVB body burdens is shown in Fig. 6B. Sitaxentan exhibited exposure-adjusted safety signals in all assays and against all endpoints, whereas bosentan exhibited a single exposure-adjusted safety
signal (for BSEP inhibition) and ambrisentan exhibited no exposure-adjusted signals.

Bosentan was evaluated clinically for treatment of hypertension at a range of doses and dose schedules (100, 500, 1000 mg q.d., or 1000 mg b.i.d.), which included doses that were markedly higher than that recommended subsequently for therapeutic use. This revealed a clear relationship between in vivo plasma drug exposure and the frequency of serum ALT elevations; furthermore, a reduced frequency of ALT elevations and reduced bosentan $C_{\text{max}}$ were observed at 1000 mg b.i.d. when compared with 1000 mg q.d. (Supplemental Table 3) (Fattinger et al., 2001), which is likely to be a consequence of enhanced clearance arising from CYP3A4 induction (Dingemanse and van Giersbergen, 2004). The maximum human plasma bosentan concentrations that were determined at the different dose levels ($C_{\text{4hr,ss}}$) (Dingemanse and van Giersbergen, 2004) or $C_{\text{4hr,ss}}/IC_{50}$ or $C_{\text{4hr,ss}}/EC_{50}$ ratios for each of the assay endpoints, whereas the drug doses were used to calculate a range of corresponding CVB body burdens (Supplemental Table 3). Exposure-adjusted assay ratios $>0.1$ were observed for BSEP inhibition when bosentan was given at doses of 500 mg, 1000 mg q.d., or 1000 mg b.i.d., but not at a dose of 100 mg, and were not observed for any of the other assay endpoints. A plot of the estimated $C_{\text{4hr,ss}}$/BSEP $IC_{50}$ ratios against CVB body burden (Fig. 6C) demonstrated an apparent correlation between these two in vitro findings and the frequency of ALT elevations observed in the bosentan clinical trial.

Discussion

Our in vitro investigations of the ERAs have shown that sitaxentan, which was withdrawn from clinical use due to its association with rare acute liver failure, displayed multiple adverse properties that have been implicated in initiation of DILI caused by other drugs. These were inhibition of the biliary efflux transporters BSEP and MRP2, metabolic bioactivation, and CVB to human hepatocyte proteins, P450-independent cell cytotoxicity, CYP3A4-potentiated cell toxicity, and impaired mitochondrial respiration. These findings suggest that the human DILI caused by sitaxentan is likely to arise as a consequence of multiple contributory mechanisms. Markedly lower potencies of these effects were exhibited by bosentan and ambrisentan, which have not been reported to cause human liver failure and continue to be used clinically.

The BSEP inhibition data that we obtained with bosentan are consistent with in vitro results obtained by other investigators (Fattinger et al., 2001; Mano et al., 2007; Dawson et al., 2012; Morgan et al., 2013) and with the elevated serum bile acid concentrations observed following bosentan administration to rats and humans, which indicate impaired BSEP-mediated bile flow in vivo (Fattinger et al., 2001). BSEP inhibition by sitaxentan and ambrisentan has been less widely studied, as have the effects of all three compounds on MRP2 activity. Modest inhibition of BSEP-mediated biliary efflux of radiolabeled taurocholate from isolated sandwich-cultured human hepatocytes was observed in the presence of 100 $\mu$M sitaxentan or bosentan, but not ambrisentan (Hartman et al., 2010). In the same study, ambrisentan did not affect MRP2-mediated biliary

**Table 2**

Covalent binding of ERAs to human hepatocyte proteins

<table>
<thead>
<tr>
<th>Drug</th>
<th>CVB</th>
<th>Turnover</th>
<th>$f_{\text{CVB}}$</th>
<th>Dose</th>
<th>CVB Body Burden</th>
</tr>
</thead>
<tbody>
<tr>
<td>$[^{4}\text{H}]$Sitaxentan$^a$</td>
<td>216</td>
<td>20.4</td>
<td>0.073</td>
<td>100, q.d.</td>
<td>100, q.d.</td>
</tr>
<tr>
<td>$[^{7}\text{H}]$Sitaxentan$^b$</td>
<td>207</td>
<td>31.6</td>
<td>0.046</td>
<td>100, q.d.</td>
<td>100, q.d.</td>
</tr>
<tr>
<td>Sitaxentan$^c$</td>
<td>212</td>
<td>26.0</td>
<td>0.060</td>
<td>100, q.d.</td>
<td>5.95</td>
</tr>
<tr>
<td>Bosentan$^d$</td>
<td>19.4</td>
<td>21.7</td>
<td>0.0061</td>
<td>125, b.i.d.</td>
<td>1.53</td>
</tr>
<tr>
<td>Ambrisentan</td>
<td>7.3</td>
<td>15.0</td>
<td>0.0034</td>
<td>10, q.d.</td>
<td>0.03</td>
</tr>
</tbody>
</table>

$^a$Due to difficulties with the radiochemical stability, this assay was repeated several times. Results from the run with the highest radiochemical purity (88%) are reported in the table.

$^b$The radiolabel purity was 89%.

$^c$The average values for $[^{4}\text{H}]$ and $[^{7}\text{H}]$sitaxentan were used in these calculations.

$^d$Average value obtained from two separate experiments.
efflux of the probe substrate [2D-penicillamine, 5-D-penicillamine] encephalin, whereas bosentan and sitaxentan exhibited a concentration-dependent decrease in only one of three hepatocyte donors. Conversely, sitaxentan and bosentan were reported to stimulate accumulation of the probe substrate estradiol-17β-glucuronide into membrane vesicles from MRP2-transfected Sf9 cells (Mano et al., 2007; Morgan et al., 2013). This contrasts with our observation of highly reproducible and concentration-dependent inhibition of MRP2-mediated accumulation of CDF by sitaxentan and bosentan, and stimulation of CDF accumulation by ambrisentan. Differences in the model systems, probe substrates, and assay conditions may explain the apparent discrepancies between our findings and those of others. Cultured primary hepatocytes express a more physiologic complement of transporter proteins than membrane vesicles from transfected insect cells, and both sitaxentan and

Fig. 5. Effects of ERAs on the viability of THLE-Null and THLE-3A4 cells incubated with varying concentrations of (A) sitaxentan, (B) bosentan, and (C) ambrisentan for 24 hours. Cell viability was determined using the MTS assay. Data were normalized to DMSO vehicle values, n = 3.

Fig. 6. Zone plots of in vitro safety data versus CVB body burden. (A) Aggregated in vitro safety scores plotted against CVB body burden values at the maximum prescribed therapeutic doses of the drugs. (B) Aggregated exposure-adjusted safety scores plotted against CVB body burden values estimated at maximum prescribed therapeutic doses. (C) Influence of bosentan dose and plasma exposure on BSEP C4h,ss/IC50 ratios and CVB body burdens. The frequency of liver injury, as defined by ALT > 3× upper limit of normal, is highlighted for each dose.
bosentan have been shown to inhibit hepatic uptake transporter activity (Lepist et al., 2014), which perhaps could mask their effects on BSEP- or MRP2-mediated biliary probe substrate efflux from hepatocytes. Differences between effects of other drugs on MRP2 activity have been observed, especially when different probe substrates were used, and have been attributed to the presence of different substrate binding sites (Kidron et al., 2012).

The Seahorse flux analyzer assay data revealed relatively potent impairment of mitochondrial respiration in cells exposed to sitaxentan. Although our experiments do not enable precise elucidation of the underlying mechanism, the effects of sitaxentan on MRR were more marked and more potent than those on OCR. This is suggestive of inhibition of one or more components of the respiratory chain, although stimulation of substrate utilization and/or destabilization of the mitochondrial membrane are also conceivable (Brand and Nicholls, 2011) and cannot be excluded.

Inhibition of mitochondrial respiration provides a plausible explanation for the marked P450-independent THLE-Null cell toxicity of sitaxentan. This drug also exhibited markedly potentiated toxicity to THLE-3A4 cells. Potentiating toxicity to THLE-3A4 cells has been observed with numerous other drugs that are bioactivated to reactive intermediates via CYP3A4 (Thompson et al., 2012; Foster et al., 2013; Gustafsson et al., 2014). Sitaxentan is metabolized by CYP3A4 (Erve et al., 2013) and exhibited a high in vitro fCIVB to human hepatocytes; therefore, a likely explanation for the potentiated toxicity to THLE-3A4 cells is the formation of a cytotoxic reactive intermediate in these cells. Investigations of sitaxentan bioactivation in human liver microsomes revealed time-dependent inhibition of CYP3A4 and formation of an unstable metabolite that was trapped by addition of reduced GSH (Erve et al., 2013). The GSH adduct was proposed to be formed via an ortho-quinone intermediate, and two potential sites for the formation of such an intermediate were proposed, one of which was more thermodynamically favored (Supplemental Scheme 1). To assess whether these sites of reactivity could explain the CVB seen in our studies, we tritium-labeled sitaxentan at both the [4-²H] and [7-²H] positions (see Fig. 1). High levels of CVB were observed in our experiments with both radioisomers, indicating no major difference in the reactivity of the two radioisomers. However, if the mechanism of CVB had proceeded via a quinone, we would have expected to see the formation of tritiated water due to the loss of the tritium label, which was not the case. Our results are thus not in agreement with the proposed role of a quinone intermediate, but rather suggest the involvement of an alternative bioactivation route. Furthermore, in view of the lack of effect on toxicity of GSH depletion by BSO pretreatment of THLE-3A4 cells, we consider that the potentiated cytotoxicity of sitaxentan in this cell line cannot plausibly be attributed to the formation of a GSH-reactive ortho-quinone metabolite.

The CVB data also provided evidence that bosentan is bioactivated to a reactive intermediate, albeit to a markedly lesser extent than sitaxentan. This offers a possible explanation for the small potentiation of toxicity that was observed following exposure to 300 µM bosentan of THLE-3A4 cells, when compared with THLE-Null cells. Bosentan is metabolized by CYP3A4 (Dingemanse and van Giersbergen, 2004), as is ambrisentan (Richards et al., 2009). The zone plots shown in Fig. 6 provide a convenient approach for visualization of the multiple assay endpoints. The plot shown in Fig. 6A is based on a previously proposed data integration, which incorporated similar in vitro assay endpoints and took account of daily therapeutic drug dose (plus the extent of in vitro metabolic turnover) when estimating CVB body burden (Thompson et al., 2012). In that study, 27 drugs that caused DILI and/or other serious nonpharmacologically mediated human adverse reactions were all distributed in zones 2, 3, or 4, whereas five of seven “safe” drugs were located in zone 1. This visualization is well suited to use in drug discovery, when chemical choice is available and it is important to select test compounds for clinical progression that have the least possible propensity to cause human toxicity. Had such an evaluation been undertaken prior to clinical evaluation of the ERAs, it would have predicted that ambrisentan has a good in vitro safety profile, sitaxentan has a poor in vitro safety profile, and bosentan has a suboptimal profile. This can be contrasted with the absence of liver injury caused by sitaxentan in animals (Owen et al., 2012).

An important limitation of this approach is that in vivo drug exposure is not taken into account when identifying and aggregating scores for the various in vitro safety assays (y-axis). Consequently, the impact of in vitro safety data obtained with low-exposure drugs could be overestimated and that of high-exposure drugs underestimated. The exposure-adjusted zone plot illustrated in Fig. 6B addresses this limitation and illustrates the apparent inter-relationship between in vitro safety assay data, in vivo doses/exposures, and the clinical safety

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**Table 3**

Exposure-adjusted in vitro toxicity data

The impact of exposure on the binary safety assay scores was assessed by estimating the ratio between the reported total steady state plasma concentration (Cmax,ss) of the drugs at the maximum prescribed doses and the observed in vitro IC50/EC50 values.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Clinical Dose Schedule</th>
<th>Cmax,ss</th>
<th>Parameter Score</th>
<th>BSEP Cmax,ss /IC50</th>
<th>MRP2 Cmax,ss /EC50</th>
<th>OCR Cmax,ss /IC50</th>
<th>MRR Cmax,ss /EC50</th>
<th>THLE-Null Cmax,ss /IC50</th>
<th>THLE-3A4 Cmax,ss /IC50</th>
<th>Exposure-Adjusted Aggregate Safety Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sitaxentan</td>
<td>100, q.d.</td>
<td>28.6</td>
<td>Value Score</td>
<td>2.75</td>
<td>0.47</td>
<td>0.21</td>
<td>0.47</td>
<td>0.18</td>
<td>0.32</td>
<td></td>
</tr>
<tr>
<td>Bosentan</td>
<td>125, b.i.d.</td>
<td>4.2</td>
<td>Value Score</td>
<td>0.15</td>
<td>0.02</td>
<td>&lt;0.02</td>
<td>0.02</td>
<td>&lt;0.01</td>
<td>0.02</td>
<td>6</td>
</tr>
<tr>
<td>Ambrisentan</td>
<td>10, q.d.</td>
<td>3.0</td>
<td>Value Score</td>
<td>0.01</td>
<td>&lt;0.003</td>
<td>&lt;0.02</td>
<td>&lt;0.02</td>
<td>&lt;0.01</td>
<td>&lt;0.02</td>
<td>0</td>
</tr>
</tbody>
</table>

Notes:

* Cmax,ss values were converted to molar concentrations (µM) from mass percentage concentrations (e.g., ng/ml), which were obtained from scientific publications (see References).

* Ratios ≥0.1 were given a score of 1, and ratios <0.1 were assigned a score of 0. Where IC50 or EC50 values were not defined (e.g., >300 µM), the highest tested compound concentrations were used to calculate ratios.
profiles of the three ERAs. Ambrisentan is situated in a low-risk zone due to a combination of low therapeutic dose (10 mg q.d.) and absence of concerning in vivo signals. Sitaxentan could be regarded as a moderate dose compound (100 mg q.d.), but is in a high-risk zone due to the number and relative potencies of in vitro adverse effects on key cell functions and health. The daily dose of bosentan is markedly higher than that of sitaxentan, yet fewer and less potent in vitro adverse properties were identified for the former drug. Furthermore, a clear association was observed between exposure-adjusted BSEP inhibition, CVB body burden, and the frequency of ALT elevations detected in a clinical trial of bosentan at doses that ranged between 100 and 1000 mg (Fattiger et al., 2001) (Fig. 6C). Overall, these data provide a credible explanation for the reduced propensity of bosentan to cause clinically concerning human DILI, when compared with sitaxentan. They also suggest that the infrequent cholestatic human DILI observed in patients treated with bosentan may not be due to BSEP inhibition alone, but rather may arise due to a combination of BSEP inhibition, metabolic bioactivation, and also its substantial daily dose.

Moreover, drug concentrations within liver cells in vivo may differ markedly from those present in peripheral blood plasma (Chu et al., 2013). For the ERAs, an in vitro study has shown accumulation of ambrisentan, bosentan, and sitaxentan in isolated hepatocytes that results in intracellular concentrations that are 4-, 20-, and 40-fold greater than extracellular concentrations, respectively (Lepist et al., 2014). If intrahepatic accumulation were to occur in vivo to similar or greater extents, this could amplify any adverse effects in the liver. The potential for intrahepatic accumulation of bosentan further highlights the impact of its relatively high therapeutic dose as an important contributor to its suboptimal human DILI risk profile.

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Wrote or contributed to the writing of the manuscript: Kenna, Stahl, Eakins, Foster, Andersson, Biliger, Elebring, Thompson.

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