Multiple compound related adverse properties contribute to liver injury caused by endothelin receptor antagonists

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Nonstandard abbreviations  AA, antimycin A; ALT, alanine aminotransferase; BSEP, bile salt export pump; BSO, buthionine sulfoximine; CDF, 5(6)-carboxy-2',7'-dichlorofluorescein; CVB, covalent binding; CYP, cytochrome P450; C max, ss, maximal drug plasma concentration at steady state; DMEM, Dulbecco’s Minimal Eagle Medium; DILI, drug-induced liver injury; ECAR, extracellular acidification rate; ERA, endothelin receptor antagonist; FCCP, carbonyl-cyanide-4-(trifluoromethoxy)phenylhydrazone; f CVB, fraction of metabolism leading to covalent binding; MRP2, multidrug-resistance associated protein 2; MRR, maximal respiration rate; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; OCR, oxygen consumption rate; PMFR, Basal Pasadena Foundation for Medical Research; rot, rotenone; THLE cells, SV40-T-antigen-immortalized human liver epithelial cells.

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Abstract

Drug-induced liver injury (DILI) 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 (MRP2) was quantified using membrane vesicle assays. Inhibition of mitochondrial respiration in human liver-derived HuH-7 cells was determined using a Seahorse XF96 analyzer. Cytochrome P450 (CYP)-independent and CYP-mediated cell toxicity was assessed using transfected SV40-T-antigen-immortalized human liver epithelial (THLE) cells lines. Exposure adjusted assay ratios were calculated by dividing the maximum human drug plasma concentrations (C_{max,ss}) by the IC_{50} or EC_{50} values obtained in vitro. Covalent binding (CVB) of radiolabelled drugs to human hepatocytes was quantified 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 5 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 which have been implicated in the development of DILI include covalent and non-covalent 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 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 non-genetic factors (Yuan and Kaplowitz, 2013).

Endothelin receptor antagonists (ERAs) are an important class of drugs where 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 hepatitis-like drug reactions, which were not evident at the time of registration and included several instances of acute liver failure, were observed post-marketing in patients treated with sitaxentan which led to the withdrawal of the drug in 2010 (Galiè 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 (Eriksson et al., 2011; Humbert et al., 2007). 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, or to cause DILI post-licensing (even incohorts of patients who previously had discontinued bosentan or sitaxentan due to plasma ALT elevations) (Frampton, 2011; McGoon et al., 2009). Consequently, although ambrisentan initially was issued a DILI black box warning by the FDA, this was subsequently removed (United States Food and Drug Administration, 2011).

The mechanisms which 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 multi-parametric assay panel, which was based on that described by Thompson et al (Thompson et al., 2012) and included assessment of biliary transporter inhibition, impairment of mitochondrial function, toxicity to cell lines expressing no human cytochrome P450 (CYP) enzymes or individual human CYP 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 \(C_{\text{max,ss}}\) by the in vitro IC\(_{50}\) or EC\(_{50}\) values.
MATERIALS AND METHODS

Materials and Chemicals. Compound structures, including the positions of the radiolabels, are shown in Fig. 1. Bosentan (Ro 47-0203, 4-tert-butyl-N-[6-(2-hydroxy-ethoxy)-5-(2-methoxy-phenoxo)-2,2’-bipyrimidin-4-yl]-benzenesulfonamide) (Clozel et al., 1994) was synthesized by Medicinal Chemistry, AstraZeneca. Sitaxentan (TBC11251, N-(4-chloro-3-methyl-1,2-oxazol-5-yl)-2-[2-(6-methyl-2H-1,3-benzodioxol-5-yl)acetyl]thiophene-3-sulfonamide) (Wu et al., 1997) and ambrisentan (BSF208075, LU208075, (2S)-2-[(4,6-dimethylpyrimidin-2-yl)oxy]-3-methoxy-3,3-diphenylpropanoic acid) (Riechers et al., 1996) were obtained from Chemtronica AB (Stockholm, Sweden). THLE cell lines (1A2, 2C9, 2C19, 2D6, 3A4 and Null) were obtained from Nestec Ltd (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 (ABCB11) was provided by Prof. Bruno Stieger (University of Zurich, Switzerland). CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay™ (MTS) was obtained from Promega UK Ltd (Southampton, UK). BioCoat™ Collagen-I Cellware 96-well plates were obtained from Becton Dickinson (Bedford, UK). [3H]taurocholic acid (1 mCi/ml) was purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA). 5(6)-carboxy-2’,7’-dichlorofluorescein (CDF), Dulbecco’s Modified Eagles Media (DMEM), unbuffered DMEM, carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP), rotenone, oligomycin, antimycin A and all other reagents were obtained from Sigma-Aldrich Company Ltd (Poole, UK). Basal Pasadena Foundation for Medical Research Medium-4 (PMFR4) medium was obtained from Invitrogen, (Paisley, UK). XF96 sensor cartridges, XF96-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]acetyl]thiophene-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]acetyl]thiophene-3-sulfonamide),
yl)acetyl]thiophene-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)oxy]-3-methoxy-3-phenyl-[4-3H]phenylpropanoic acid) (see Fig. 1) was undertaken in two steps by a halogenation-tritiodehalogenation process. Halogenation of sitaxentan was effected using 1 equivalent of N-iodosuccinimide in 10% trifluoroacetic acid in dichloromethane. Two regioisomeric iodides were separated by preparative HPLC and were identified by Heteronuclear Multiple Bond Correlation NMR 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 was accomplished using standard methodology (T₂, Pd-CaCO₃, Hunig’s base with EtOH as solvent), and the crude tritium labeled compounds were purified by preparative HPLC. The radiochemical purities of [7-3H]sitaxentan, [4-3H]sitaxentan, [3H]bosentan and [3H]ambrisentan were determined by HPLC to be 91%, 98%, 98% and 99%, respectively, and the specific activities were determined by LCMS to be 270 kBq/nmol, 700 kBq/nmol, 980 kBq/nmol, 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 re-purified the day prior to use and a stock solution of the compound in 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 MRP2 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 MRP2 proteins were quantified using rapid filtration assays in 96-well plate format, as described by Rodrigues et al.(Rodrigues et al.,
The BSEP substrate was \(^{[3H]}\)taurocholate (PerkinElmer, Waltham, MA) and the MRP2 substrate was 5(6)-carboxy-2',7'-dichlorofluorescein (CDF) (Sigma-Aldrich, Poole, UK). 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 XF-96 flux analyzer (Seahorse Biosciences), as described by Brand and Nicholls (Brand and Nicholls, 2011). The cells were exposed to the ERA compounds, following which mitochondrial stress was evaluated by subsequent consecutive addition of oligomycin, carbonyl-cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP) and rotenone plus antimycin A (Rot/AA), according to the manufacturer’s guidelines.

Cells which had been cultured at 37°C in tissue culture flasks in DMEM (D6429) supplemented with 10% FBS, in 5% CO\(_2\):95% air, were seeded at 20,000 cells/well onto XF-96 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 (BSA) (medium pH 7.4, 37°C) immediately prior to each assay and cells were then incubated in a CO\(_2\) free incubator at 37°C for 90 min. Basal rates of oxygen consumption (OCR) were determined on three sequential readings, each comprising a 3 min mix and 4 min read cycle. Concentrated solutions (10x) of the ERA compounds or dimethylsulfoxide (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 a further 6 cycles and also following further addition of oligomycin (1 μM; Injection 2), carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP, 1.5 μM; Injection 3) and rotenone and antimycin A (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
EC$_{50}$ values, non-mitochondrial 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 Maximal Respiration Rate (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 nM hydrocortisone, 0.5 ng/mL epidermal growth factor, 35 µg/mL bovine pituitary extract (BPE), 0.33nM retinoic acid, 3% foetal bovine serum (FBS), 150 µg/mL G418), in BioCoat™ Collagen-I 96-well plates, and were exposed for 18 h to the γ-glutamylcysteine synthetase inhibitor buthionine sulfoximine (BSO; 80 µM) in PFMR4(-) medium (basal PMFR(+)) medium without insulin, transferrin, sodium selenite or FBS), or to PFMR4(-) 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 a further 24 h. 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, Southampton, UK).

**Covalent Binding (CVB) Studies in Human Hepatocytes.** Studies were undertaken in pooled cryopreserved human hepatocytes using the semi-automated 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 [4-³H]- and [7-³H]-labeled sitaxentan (500 µM) were prepared directly in DMSO which contained unlabeled compound, acetonitrile and water. Hepatocyte suspensions (585 µL, 1,000,000 cells/mL) were pre-incubated for 5 min at 37ºC, incubations were initiated by the addition of pre-heated radiolabeled compounds (65 µL) and were performed for 4 h. The final DMSO and acetonitrile concentrations were 0.1% and 1%, respectively, and all incubations were carried out in
triplicate. After additon of test compound and at the end of the incubation (i.e. at 0 minutes and 4 hours, respectively) aliquots were taken for CVB and turnover determination (200 and 50 µL, respectively). To account for non-specific binding, the net covalent binding of each compound (CVB_{net}) was determined by subtracting its 0 min value (CVB_{T=0}) from the paired result after the 4 h incubation (CVB_{T=end}). 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 Corporation, Milford, MA). A Sunniest RP-AQUA HPLC (3 µm, 2.1x30 mm) column was used with the column heater set at 40°C. For elution, a generic gradient was used from 96% A (H₂O containing 0.2% formic acid) and 4% B (acetonitrile containing 0.2% formic acid) to 95% B over 1.2 minutes. Chromatographic peaks corresponding to the parent drug molecules were quantified by QuanLynx software (Waters Corporation) using a calibration curve. The difference in concentration for the parent drug between t = 0 h and t = 4 h was compared to obtain % turnover estimate for the test compound. To identify possible formation of ³H₂O the samples were analyzed with LC-RAD. Samples were analyzed on an Agilent 1100 series HPLC system (Agilent Technologies) coupled to an ARCTM (AIM Research Company, Hockessin, DE). A Waters xBridge C18 HPLC (2.5 µm, 2.1x50 mm) column was used with the column heater set at 40°C. For elution, a generic gradient was used from 98% A (H₂O containing 0.2% formic acid) and 2% B (acetonitrile containing 0.2% formic acid) to 95% solvent B over a 9 minute using a linear gradient. LC-RAD data are processed using the ARCTM Evaluation software (AIM Research Company, Hockessin, DE).

The fraction of metabolism leading to covalent binding (f_{CVB}) was calculated from the net covalent binding (CVB_{net}), amount of protein in the incubation, amount of compound and fractional turnover of the parent compound, as described previously (Thompson et al., 2012). The CVB Burden was calculated using CVB Burden = D x f_{CVB}, where D is the maximum prescribed daily dose (mg/day).

**Statistical Analyses.** Data are expressed as means ± SEM. IC₅₀ and EC₅₀ values were determined by non-linear regression using GraphPad Prism (GraphPad Software, La Jolla, CA) and are expressed as
 RESULTS

 Effects of ERAs on BSEP and MRP2 activities. Each of the ERAs caused concentration-dependent inhibition of [3H]taurocholate uptake by membrane vesicles which expressed human BSEP. Sitaxentan was the most potent and ambrisentan the least potent BSEP inhibitor (Fig. 2A; Table 1; and Supplemental Table 1). Sitaxentan and bosentan also caused concentration-dependent inhibition of CDF uptake by membrane vesicles which 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 (Supplemental Fig. 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 XF96-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), i.e. 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 Maximal Respiration Rate (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 (Fig. 3A; Fig. 4A,B; Table 1 and Supplemental 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). However, no effects on either OCR or MRR were observed at lower bosentan concentrations (Fig. 3B; Fig. 4A,B; 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 (Fig. 3C; Fig. 4A,B; Table 1). This was the maximum concentration of the compound that could be achieved in the assay medium utilized in the XF96-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 CYP 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 CYP activities (THLE-Null cells) or the major human hepatic CYP enzymes CYP1A2, CYP2C9, CYP2C19, CYP3A4 or CYP2D6. The value of these cell lines for investigation of CYP independent and CYP 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 h (Fig. 5A; Table 1; Supplemental 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 EC\textsubscript{50} ratio of 1.8. Bosentan caused concentration-dependent effects on cell viability when tested at concentrations which exceeded 100 µM and under these conditions there was also a small increase in toxicity to THLE-3A4 cells, compared to 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 which expressed alternative human hepatic CYP enzymes (CYP1A2, CYP2C9, CYP2C19, CYP2D6) were similar to those observed in THLE-Null cells (Supplemental Fig. 3-5; Supplemental Table 2). The potencies of toxicity of the ERAs to THLE cell lines were unaffected by cell exposure to BSO (Supplemental Fig. 3-5; Supplemental Table 2). BSO is a potent inhibitor of γ-glutamylcysteine synthetase, 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 radiolabelled ERAs to human hepatocyte proteins.** CVB studies with sitaxentan were performed using two tritium-labeled analogues, ([4-\textsuperscript{3}H]sitaxentan and [7-\textsuperscript{3}H]sitaxentan; the chemical structures of these and the other radiolabelled ERAs are shown in Fig. 1. Both sitaxentan analogues 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 tritiated water was seen with any of the compounds. In order 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 analogues 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 on the other hand, 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 or not 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 which caused human DILI and by 5 of 21 (24%) drugs which did not cause DILI (Dawson et al., 2012). The threshold value for Mrp2 inhibition (≤300 µM) was exhibited by 8 of 30 drugs which caused human DILI (27%) and by 0 of 6 drugs (0%) which 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 which caused human DILI (26%) and by 1 of 21 drugs (5%) which 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 which caused DILI (12%) and by 1 of 21 drugs (5%) which did not (Gustafsson et al., 2014).

Since comparative reference mitochondrial respiration assay data obtained with toxic and non-toxic 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, while bosentan and ambrisentan exhibited considerably 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 4 zones, as proposed by Thompson et al. following analysis of 36 drugs (Thompson et al., 2012). 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, 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_{max,ss}) of the drugs (Croxtall and Keam, 2008; Dingemanse and van Giersbergen, 2004; Scott, 2007) 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, (United States Food and Drug Administration, 2012) a 10-fold safety margin was used and ratios \geq 0.1 were assigned scores of 1, while ratios <0.1 were assigned scores of 0. Calculation of the ratios between C_{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_{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 are 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 mg, 500 mg, 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_{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 which were determined at the different dose levels (C₄hr,ss) (Dingemanse and van Giersbergen, 2004) were used to calculate C₄hr,ss/IC₅₀ or C₄hr,ss/EC₅₀ ratios for each of the assay endpoints, while 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 C₄hr,ss/BSEP IC₅₀ 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 which 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, CYP-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 we obtained with bosentan are consistent with in vitro results obtained by other investigators (Dawson et al., 2012; Fattinger et al., 2001; Mano et al., 2007; 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 radiolabelled taurocholate from isolated sandwich-cultured human hepatocytes was observed in the presence of 100 µM sitaxentan or bosentan, but not ambrisentan (Hartman et al., 2010). In the same study, ambrisentan did not affect MRP2-mediated biliary efflux of the probe substrate [2D-penicillamine,5-D-penicillamine]encephalin, while bosentan and sitaxentan exhibited a concentration-dependent decrease in only one out 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 physiological complement of transporter proteins than membrane vesicles from transfected insect cells and both sitaxentan and 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 CYP-independent THLE-Null cell toxicity of sitaxentan. This drug also exhibited markedly potentiated toxicity to THLE-3A4 cells. Potentiated toxicity to THLE-3A4 cells has been observed with numerous other drugs which are bioactivated to reactive intermediates via CYP3A4 (Foster et al., 2013; Gustafsson et al., 2014; Thompson et al., 2012). Sitaxentan is metabolized by CYP3A4 (Erve et al., 2013) and exhibited a high in vitro fCVB 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 biotransformation in human liver microsomes revealed time-dependent inhibition of CYP3A4 and formation of an unstable metabolite that was trapped by addition of reduced glutathione (Erve et al., 2013). The glutathione 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. To assess if these
sites of reactivity could explain the CVB seen in our studies, we tritium-labeled sitaxentan at both the 
$[4-^3\text{H}]$- and $[7-^3\text{H}]$-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 pre-treatment 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 glutathione 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 which caused DILI and/or other serious non-pharmacologically mediated human 
adverse reactions (ADRs) were all distributed in Zones 2, 3 or 4, whereas 5 of 7 “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,

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sitaxentan has a poor in vitro safety profile and bosentan has a sub-optimal 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 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 vitro 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 which ranged between 100 mg and 1000 mg (Fattinger 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 which 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.
Acknowledgments

We thank Bruno Stieger for provision of hBSEP-expressing baculovirus stocks, Johan E. Palm for provision of the hMRP2-encoding plasmid, Laura Liggins for membrane vesicle preparation and Paul Morgan for valuable discussions.

Authorship Contributions

*Participated in research design*: Kenna, Stahl, Billger, Elebring, Thompson

*Conducted experiments*: Stahl, Eakins, Foster, Andersson, Bergare, Elmore

*Performed data analysis*: Kenna, Stahl, Eakins, Foster, Andersson, Elmore, Thompson

*Wrote or contributed to the writing of the manuscript*: Kenna, Stahl, Eakins, Foster, Andersson, Billger, Elebring, Elmore, Thompson
References


United States Food and Drug Administration. (2011) FDA drug safety communication: Liver injury warning to be removed from letairis (ambrisentan) tablets. 2014.


Figure Legends

Fig. 1. Structures and positions of tritium in radiolabelled ERAs. For each compound the position of the radiolabel has been indicated by use of the symbol “T”.

Fig. 2. Effects of ERAs on biliary efflux transporter activity, determined via ATP-dependent (A) [3H]taurocholate (TCA) uptake by vesicles expressing BSEP, n=4, and (B) CDF uptake by vesicles expressing MRP2, n≥3. All values are expressed as a percentage of DMSO vehicle control.

Fig. 3. Effects of ERAs on cellular respiration in HuH-7 cells exposed sequentially to ERAs or DMSO vehicle alone (Injection 1), oligomycin (Injection 2), FCCP (Injection 3) and Rot/AA (Injection 4). OCR % normalized to pre-dose OCR shown following administration of (A) sitaxentan, (B) bosentan or (C) ambrisentan. The white box indicates the time point following injection of ERA or DMSO vehicle that was used to determine EC50 values for OCR. The grey shaded box indicates the time point following injection of FCCP that was used to quantify effects on MRR.

Fig. 4. Concentration-response curves for (A) OCR, n=4, and (B) MRR, n=4, each expressed as % normalized to DMSO vehicle control.

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 h. 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 vs. 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 $C_{\text{th,so}}$IC$_{50}$ ratios and CVB body burdens. The frequency of liver injury, as defined by ALT > 3xULN, is highlighted for each dose.
TABLES

**Table 1.** Effects of ERAs on in vitro toxicity endpoints. Assessment of effects of ERAs on biliary transport (BSEP, MRP2), mitochondrial function (OCR, MRR), non-CYP and CYP3A4-mediated cell toxicity (THLE-NULL and THLE-Null/3A4 ratio). Values are means from independent replicate experiments, n≥3.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Parameter</th>
<th>Safety assay endpoint</th>
<th>Aggregate safety score</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BSEP IC₅₀</td>
<td>MRP2 IC₅₀</td>
<td>Basal OCR</td>
</tr>
<tr>
<td></td>
<td>(μM)</td>
<td>(μM)</td>
<td>EC₅₀ (μM)</td>
</tr>
<tr>
<td>Sitaxentan</td>
<td>Value</td>
<td>10.4</td>
<td>61.0</td>
</tr>
<tr>
<td></td>
<td>Score</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Bosentan</td>
<td>Value</td>
<td>28.0</td>
<td>215.1</td>
</tr>
<tr>
<td></td>
<td>Score</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Ambrisentan</td>
<td>Value</td>
<td>288.1</td>
<td>&gt;1000</td>
</tr>
<tr>
<td></td>
<td>Score</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>
Assessment of effect of ERAs on biliary transport (BSEP, MRP2), mitochondrial function impairment (OCR, MRR), non-CYP and CYP3A4 mediated cell toxicity (THLE-NULL and THLE-Null/3A4 ratio). Compounds were classed as active if the IC$_{50}$/EC$_{50}$ values were ≤300 µM, BSEP and MRP2; ≤200 µM OCR, MRR; ≤200 µM THLE-Null; ≥1.4 THLE-Null/3A4 ratio.
Table 2. Covalent binding of ERAs to human hepatocyte proteins. Covalent binding to protein was determined following incubation of bosentan (10 mM), ambrisentan (10 mM) and sitaxsentan (500 µM) with pooled cryopreserved human hepatocytes for 4 h. CVB body burdens were calculated by multiplying fCVB by the maximum recommended daily therapeutic doses of the drugs. The CVB body burden threshold of safety concern was ≥1 mg/day. Ambrisentan was tested in three separate experiments. In all instances the observed turnover of the compound was low and it could be quantified only on one occasion. This value is shown.

<table>
<thead>
<tr>
<th>Drug</th>
<th>CVB (pmol drug eq./mg protein)</th>
<th>Turnover (%)</th>
<th>$f_{CVB}$</th>
<th>Dose (mg)</th>
<th>CVB body burden (mg/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[4-3H]Sitaxentan$^a$</td>
<td>216</td>
<td>20.4</td>
<td>0.073</td>
<td>100, q.d.</td>
<td></td>
</tr>
<tr>
<td>[7-3H]Sitaxentan$^b$</td>
<td>207</td>
<td>31.6</td>
<td>0.046</td>
<td>100, q.d.</td>
<td></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 [4-3H] and [7-3H]sitaxentan were used in these calculations.

$^d$ Average value obtained from two separate experiments.
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 ($C_{\text{max,ss}}$) of the drugs at the maximum prescribed doses and the observed in vitro IC$_{50}$/EC$_{50}$ values.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Clinical dose (mg) and dose schedule</th>
<th>$C_{\text{max,ss}}$ (μM)$^a$</th>
<th>Parameter</th>
<th>Endpoint</th>
<th>Exposure adjusted aggregate safety score</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>BSEP</td>
<td>MRP2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>$C_{\text{max,ss}}$/IC$_{50}$</td>
<td>$C_{\text{max,ss}}$/IC$_{50}$</td>
</tr>
<tr>
<td>Sitaxentan</td>
<td>100, q.d.</td>
<td>28.6</td>
<td>Value</td>
<td>2.75</td>
<td>0.47</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Score$^b$</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Bosentan</td>
<td>125, b.i.d.</td>
<td>4.2</td>
<td>Value</td>
<td>0.15</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Score$^b$</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Ambrisentan</td>
<td>10, q.d.</td>
<td>3.0</td>
<td>Value</td>
<td>0.01</td>
<td>&lt;0.003</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Score$^b$</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

$^a$ $C_{\text{max,ss}}$ values were converted to molar concentrations (μM) from mass percentage concentrations (e.g. ng/mL), which were obtained from scientific publications (see references). $^b$ Ratios $≥$0.1 were given a score of 1 and ratios $<$0.1 were assigned a score of 0. Where IC$_{50}$ or EC$_{50}$ values were not defined (e.g. $>$300 μM) the highest tested compound concentrations were used to calculate ratios.
Fig. 3

A

Compound

Oligomycin

Rot/AA

OCR (% of pre-dose)

Time (min)

DMSO

62.5 µM

125 µM

250 µM

B

OCR (% of pre-dose)

Time (min)

DMSO

62.5 µM

125 µM

250 µM

C

OCR (% of pre-dose)

Time (min)

DMSO

31.3 µM

62.5 µM

125 µM
Fig. 4

A

OCR (% of vehicle control)

Concentration (µM)

Sitaxentan
Bosentan
Ambrisentan

B

MRR (% of vehicle control)

Concentration (µM)

Sitaxentan
Bosentan
Ambrisentan
Multiple compound related adverse properties contribute to liver injury caused by endothelin receptor antagonists

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Supplemental Scheme 1. Metabolism of sitaxentan as proposed by Erve et al., 2013, illustrating the two potential sites of reactivity.
Supplemental Table 1. Potencies of effects of ERAs on in vitro toxicity endpoints

<table>
<thead>
<tr>
<th>Assay</th>
<th>Parameter</th>
<th>Sitaxentan</th>
<th>Bosentan</th>
<th>Ambrisentan</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC&lt;sub&gt;50&lt;/sub&gt; (µM)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BSEP inhibition</td>
<td>10.4</td>
<td>7.4 - 14.6</td>
<td>28.0</td>
<td>288.1</td>
</tr>
<tr>
<td></td>
<td>95% CI</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>MRP2 inhibition</td>
<td>61.0</td>
<td>34.6-107.5</td>
<td>215.1</td>
<td>&gt;1000&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>95% CI</td>
<td>3</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Basal OCR</td>
<td>135.5</td>
<td>70.2 - 261.0</td>
<td>&gt;250</td>
<td>&gt;125</td>
</tr>
<tr>
<td></td>
<td>95% CI</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>MRR</td>
<td>60.6</td>
<td>36.3 - 101.0</td>
<td>211.2</td>
<td>&gt;125</td>
</tr>
<tr>
<td></td>
<td>95% CI</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>THLE-Null toxicity</td>
<td>160.0</td>
<td>96.0 – 268.0</td>
<td>&gt;300</td>
<td>&gt;300</td>
</tr>
<tr>
<td></td>
<td>95% CI</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>THLE-3A4 toxicity</td>
<td>89.0**&lt;sup&gt;b&lt;/sup&gt;</td>
<td>38.0 – 209.0</td>
<td>206.0*&lt;sup&gt;c&lt;/sup&gt;</td>
<td>&gt;300</td>
</tr>
<tr>
<td></td>
<td>95% CI</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>THLE cell toxicity</td>
<td>THLE Null/3A4</td>
<td>1.8</td>
<td>&gt;1.5</td>
<td>nd&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>EC&lt;sub&gt;50&lt;/sub&gt; (µM)</td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

<sup>a</sup> Increased MRP2 activity was observed in the presence of ambrisentan.

<sup>b</sup> ANOVA **p<0.01 statistically significantly different from THLE-Null value.

<sup>c</sup> ANOVA *p<0.05 statistically significantly different from THLE-Null value.

<sup>d</sup> nd = could not be determined.

Assessment of effect of ERAs on biliary transport (BSEP, MRP2), mitochondrial function impairment (OCR, MRR), non-CYP and CYP3A4 mediated cell toxicity (THLE-NULL, THLE-3A4 and THLE-Null/3A4 ratio). Data are mean and 95% confidence intervals (CI).
**Supplemental Table 2.** Effects of ERAs on viability of all tested THLE cell lines

Data are n=3 except for sitaxentan in the 1A2 cell line, where n=2.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Parameter</th>
<th>THLE-1A2</th>
<th>THLE-2C9</th>
<th>THLE-2C19</th>
<th>THLE-2D6</th>
<th>THLE-3A4</th>
<th>THLE-Null</th>
<th>Null/3A4 ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sitaxentan</td>
<td>EC$_{50}$ (μM)</td>
<td>229</td>
<td>181</td>
<td>178</td>
<td>231**$^a$</td>
<td>89**$^a$</td>
<td>160</td>
<td>1.8</td>
</tr>
<tr>
<td></td>
<td>95% CI</td>
<td>57-917</td>
<td>133-245</td>
<td>113-281</td>
<td>203-264</td>
<td>38-209</td>
<td>96-268</td>
<td></td>
</tr>
<tr>
<td>Bosentan</td>
<td>EC$_{50}$ (μM)</td>
<td>&gt;300</td>
<td>&gt;300</td>
<td>&gt;300</td>
<td>&gt;300</td>
<td>206$^b$</td>
<td>&gt;300</td>
<td>&gt;1.5</td>
</tr>
<tr>
<td></td>
<td>95% CI</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>91-468</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ambrisentan</td>
<td>EC$_{50}$ (μM)</td>
<td>&gt;300</td>
<td>&gt;300</td>
<td>&gt;300</td>
<td>&gt;300</td>
<td>&gt;300</td>
<td>&gt;300</td>
<td>nd$^c$</td>
</tr>
<tr>
<td></td>
<td>95% CI</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

$^a$ ANOVA **p<0.01 statistically significantly different from THLE-Null.

$^b$ ANOVA *p<0.05 statistically significantly different from THLE-Null.

$^c$ nd = could not be determined.

THLE-Null, 1A2, 2C9, 2D6, 2C19 and THLE-3A4 cell lines were incubated with varying concentrations of sitaxentan, bosentan and ambrisentan for 24 hours in the presence or absence of buthionine sulfoxamine to deplete intracellular GSH. Cell viability was assessed using the MTS assay. Data are normalized to DMSO control and are mean and 95% confidence intervals.
**Supplemental Table 3.** Influence of bosentan dose and plasma exposure on CVB burden and exposure adjusted aggregated in vitro safety scores

<table>
<thead>
<tr>
<th>Dose (mg)</th>
<th>Incidence of ALT elevations (%)</th>
<th>CVB body burden (mg/day)</th>
<th>C&lt;sub&gt;4h,ss&lt;/sub&gt; (µM)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>C&lt;sub&gt;4h,ss&lt;/sub&gt;/IC&lt;sub&gt;50&lt;/sub&gt; or C&lt;sub&gt;4h,ss&lt;/sub&gt;/EC&lt;sub&gt;50&lt;/sub&gt; for indicated endpoint</th>
<th>Exposure adjusted aggregate safety score</th>
</tr>
</thead>
<tbody>
<tr>
<td>100, q.d.</td>
<td>2</td>
<td>0.61</td>
<td>1.5</td>
<td>Value 0.05 0.01 &lt;0.01 0.07 &lt;0.01 0.01</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Score&lt;sup&gt;b&lt;/sup&gt; 0 0 0 0 0 0</td>
<td></td>
</tr>
<tr>
<td>500, q.d.</td>
<td>4</td>
<td>3.05</td>
<td>2.7</td>
<td>Value 0.10 0.01 &lt;0.01 0.01 &lt;0.01 0.01</td>
<td>1</td>
</tr>
<tr>
<td></td>
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<td></td>
<td></td>
<td>Score&lt;sup&gt;b&lt;/sup&gt; 1 0 0 0 0 0</td>
<td></td>
</tr>
<tr>
<td>1000, q.d.</td>
<td>11</td>
<td>6.10</td>
<td>4.7</td>
<td>Value 0.17 0.02 &lt;0.02 0.02 &lt;0.02 0.02</td>
<td>1</td>
</tr>
<tr>
<td>1000, b.i.d.</td>
<td>8</td>
<td>12.2</td>
<td>3.2</td>
<td>Value 0.11 0.01 &lt;0.01 0.02 &lt;0.01 0.02</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Score&lt;sup&gt;b&lt;/sup&gt; 1 0 0 0 0 0</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Plasma concentrations were determined 4 h after dosing.

<sup>b</sup> Ratios ≥0.1 were given a score of 1 and ratios <0.1 were assigned a score of 0. Where IC<sub>50</sub> or EC<sub>50</sub> values were not defined (e.g. >300 µM) the highest test concentration was used to calculate ratios.

<sup>c</sup> ANOVA *p<0.05 statistically significantly different from THLE-Null.

Ratios of the reported total steady state plasma concentrations (C<sub>max,ss</sub>) and the observed in vitro IC<sub>50</sub> or EC<sub>50</sub> values were calculated at 4 different prescribed doses of bosentan, 100 mg q.d., 500 mg q.d., 1000 mg q.d. and 1000 mg q.d. Calculation of the ratios between C<sub>max,ss</sub> and THLE-Null/THLE-3A4 EC<sub>50</sub> ratios was considered inappropriate for estimation of exposure adjusted potentiated THLE-3A4 cell toxicity and was not undertaken. Instead, C<sub>max,ss</sub>/THLE-3A4 EC<sub>50</sub> ratios were estimated.
Supplemental Figure 1. Effects on CDF uptake by vesicles expressing MRP2 were determined in the presence of AMP or ATP. Data were obtained from a representative experiment, performed in duplicate. Data for bosentan and ambrisentan are presented with circular and triangular symbols, respectively.
Supplemental Figure 2. Effects of (A) sitaxentan, (B) bosentan and (C) ambrisentan on ECAR in HuH-7 cells. Cells were incubated with varying concentrations of the ERAs (Injection 1) followed by subsequent addition of oligomycin (Injection 2), FCCP (Injection 3) and finally rotenone/antimycin A (Injection 4). Changes in ECAR were monitored in real time. Data are expressed as mean ECAR ± SEM (n=3), normalized to the pre-dose values (pre Injection 1).
Supplemental Figure 3: Effects of ERA compounds on viability in THLE cell lines. THLE-Null, 1A2, 2C9, 2D6, 2C19 and THLE-3A4 cell lines were incubated with varying concentrations of (A) sitaxentan, (B) bosentan and (C) ambrisentan for 24 hours and cell viability was assessed using MTS. Data are normalized to DMSO control and are mean ± SEM (n=3) except for ambrisentan in THLE-2C19 and sitaxentan in THLE-1A2 cell lines where (n=2).
Supplemental Figure 4. Effects of sitaxentan on viability in THLE cell lines with and without glutathione depletion. THLE cell lines were incubated with varying concentrations of sitaxentan in the presence and absence of the glutathione depleting agent BSO for 24 hours and cell viability was assessed using MTS. For the (-BSO) condition, cells were pre-treated for 18 hours with 80 μM BSO before addition of the drug. Data from incubations with THLE cell lines in presence of BSO are displayed with open symbols and in absence of BSO with closed symbols. Data are normalized to DMSO control and are mean ± SEM (n=3) except for THLE-1A2 where (n=2).
**Supplemental Figure 5.** Effects of bosentan on viability in THLE cell lines with and without glutathione depletion. THLE cell lines were incubated with varying concentrations of bosentan in the presence and absence of the glutathione depleting agent BSO for 24 hours and cell viability was assessed using MTS. For the (-BSO) condition, cells were pre-treated for 18 hours with 80 μM BSO before addition of the drug. Data from incubations with THLE cell lines in presence of BSO are displayed with open symbols and in absence of BSO with closed symbols. Data are normalized to DMSO control and are mean ± SEM (n=3).
Supplemental Figure 6. Effects of ambrisentan on viability in THLE cell lines with and without glutathione depletion. THLE cell lines were incubated with varying concentrations of ambrisentan in the presence and absence of the glutathione depleting agent BSO for 24 hours and cell viability was assessed using MTS. For the (-BSO) condition, cells were pre-treated for 18 hours with 80 μM BSO before addition the drug. Data from incubations with THLE cell lines in presence of BSO are displayed with open symbols and in absence of BSO with closed symbols. Data are normalized to DMSO control and are mean ± SEM (n=3) except THLE-2C19 –BSO where (n=2).