Hepatocyte-based in vitro model for assessment of drug-induced cholestasis

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ABSTRACT

Early detection of drug-induced cholestasis remains a challenge during drug development. We have developed and validated a biorelevant sandwich-cultured hepatocytes- (SCH) based model that can identify compounds causing cholestasis by altering bile acid disposition. Human and rat SCH were exposed (24–48 h) to known cholestatic and/or hepatotoxic compounds, in the presence or in the absence of a concentrated mixture of bile acids (BAs). Urea assay was used to assess (compromised) hepatocyte functionality at the end of the incubations. The cholestatic potential of the compounds was expressed by calculating a drug-induced cholestasis index (DICI), reflecting the relative residual urea formation by hepatocytes co-incubated with BAs and test compound as compared to hepatocytes treated with test compound alone. Compounds with clinical reports of cholestasis, including cyclosporin A, troglitazone, chlorpromazine, bosentan, ticlopidine, ritonavir, and midecamycin showed enhanced toxicity in the presence of BAs (DICI ≤ 0.8) for at least one of the tested concentrations. In contrast, the in vitro toxicity of compounds causing hepatotoxicity by other mechanisms (including diclofenac, valproic acid, amidarone and acetaminophen), remained unchanged in the presence of BAs. A safety margin (SM) for drug-induced cholestasis was calculated as the ratio of lowest in vitro concentration for which was DICI ≤ 0.8, to the reported mean peak therapeutic plasma concentration. SM values obtained in human SCH correlated well with reported % incidence of clinical drug-induced cholestasis, while no correlation was observed in rat SCH. This in vitro model enables early identification of drug candidates causing cholestasis by disturbed BA handling.

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Introduction

Cholestasis represents a pathological liver condition characterized by the impairment of bile secretion. Cholestasis is associated with accumulation of the bile acids (BAs) and other cholephiles in the liver (Fischer et al., 1996). The cytotoxicity of accumulated BAs has been implicated as one of the major causes of hepatocellular damage noted during cholestasis (Attili et al., 1986). BAs induce apoptosis at lower concentrations (in micromolar range), while they elicit necrotic damage to the cells at higher concentrations (in the millimolar range) close to the critical micelle concentration of BAs (Perez and Briz, 2009).

BA homeostasis is maintained by synchronized activity of different enzymes and transport proteins. Primary BAs (cholic acid and chenodeoxycholic acid) are synthesized from cholesterol in hepatocytes. The synthesized unconjugated BAs (e.g. cholic acid, chenodeoxycholic acid) are conjugated with either glycine or taurine and are excreted into the bile canaliculi by the bile salt export pump (BSEP/Bsep, ABCB11/Abcb11), a ATP-dependent efflux transporter. Conjugated and unconjugated BAs are further sulfated or glucuronidated in the liver. Sulfated and glucuronidated BAs are transported into the bile canalliculi by multidrug resistance associated protein-2 (MRP2/Mrp2, ABCC2/Abcc2). Multi-drug resistance protein-3 (MDR3/Mdr2, ABCB4/Abcb4) is a flavoprotein that is involved in translocation of phosphatidylcholine from the inner to the outer bilayer of the bile canalicular membrane. In the bile duct, BAs form mixed micelles after associating with cholesterol and phosphatidylcholine. The mixed micelles protect the bile duct surface from the detergent effects of the BAs (Elferink and Paulusma, 2007). In the intestine secondary BAs (deoxycholic acid, lithocholic acid, and ursodeoxycholic acid) are formed by the action of intestinal flora. BAs are taken up by the enterocytes via the apical sodium-dependent bile acid transporter (ASBT/Abgt; SLC10A2/Slc10a2). Heteromeric organic solute transporters Ostα-β, localized in the basolateral membrane of the enterocytes, effluxes the BAs to the portal circulation (Dawson et al., 2004; http://dx.doi.org/10.1016/j.taap.2013.10.032).
Compounds have demonstrated (Marion et al., 2007) inhibition of BSEP (Fattinger et al., 2001; Funk et al., 2001). Follow-up studies with these compounds has been added to the label of other drugs such as bosentan (Rodriguez-Garay, 2003). Not surprisingly, with the current biochemistry of drug-induced cholestasis, early detection of corresponding safety issues during drug development remains highly challenging. Animal models of drug-induced cholestasis, such as: (i) BA pools in humans and rodents are qualitatively and quantitatively distinct (Setchell et al., 1997), (ii) the quantitatively major BAs in rodents (taurine conjugated) are more hydrophilic and less toxic than the major BAs present in human (glycine conjugated) (Rodriguez-Gray, 2003). Not surprisingly, with the current biochemical and histological markers only 50% of the clinical cases of liver toxicity are detected in preclinical animal models (Olson et al., 2000). In addition, the in vitro testing models using human hepatocytes detected only 50–60% cases (drugs and drug candidates) of drug-induced liver injury (Xu et al., 2008).

Existing in vitro models for detecting compounds which can cause cholestasis rely on determining the extent of inhibition of BSEP-mediated taurocholate (TCA) excretion in sandwich-cultured hepatocytes (SCH) (B-CLEAR®) (Marion et al., 2007) or in BSEP/Bsep expressing vesicle models (Dawson et al., 2012; Morgan et al., 2010). These methods provide unique mechanistic information on potential interactions of drug candidate(s) with a representative BA (most often TCA) disposition in the liver. However, the limitations associated with these in vitro models are: (i) TCA is not a quantitatively important BA in human, and does not seem to play a significant role in hepatotoxicity upon its intracellular accumulation (Chatterjee et al., in press); (ii) the bioanalysis of TCA requires the use of a radiolabeled isotope or of LC-MS/MS instrumentation; (iii) multiple mechanisms are frequently involved in the toxicity exerted by a compound: even a mild inhibition of BSEP/Bsep can potentiate the existing toxicity due to concomitant reactive metabolite formation or direct mitochondrial toxicity by the compound (e.g. flutamide, ticlopidine, chlorpromazine) (Anthérien et al., 2013; Kang et al., 2008; Yoshikado et al., 2013); (iv) for some compounds e.g. troleandomycin, the metabolite (troleandomycin sulfate) is a more potent BSEP inhibitor than the parent compound (Funk et al., 2001); direct in vitro BSEP inhibition studies with these compounds alone may not reveal the full implication of BSEP inhibition in vivo; (v) basolateral efflux of BAs in the hepatocytes becomes particularly important during hindrance in their canalicular efflux. For instance if a compound also inhibits MRP3/4 (apart from BSEP), BA accumulation at supra-physiological levels and subsequent bile acid-mediated liver injury is more likely to follow. It is noteworthy that inhibition of MRP4 has recently been shown to be associated with toxicity associated with certain HIV protease inhibitors (Fukuda et al., 2013). SCH expressing the basolateral and canalicular transporters, provide us with the opportunity to investigate the effect of a xenobiotic on the overall disposition of BAs.

Clearly, evaluation of BSEP/Bsep inhibition is not sufficient to accurately predict drug-induced cholestasis for compounds exerting hepatotoxicity via multiple and/or complex mechanisms. This illustrates that there is an unmet need for a cost-effective, conceptually simple, higher-throughput in vitro model, granting reliable prediction of the liability of new drug candidates regarding drug-induced cholestasis.

We have developed a SCH-based in vitro assay to identify compounds that may cause cholestasis by interfering with BA disposition. The assay was validated using a set of known cholestatic (as positive control) and non-cholestatic but hepatotoxic compounds (as negative control) in both rat and human SCH. The clinical relevance of the assay was illustrated by demonstrating a correlation between in vitro cholestasis potential and clinical incidence data on cholestasis.

**Materials.** Williams’ E Medium (WEM), l-glutamine, penicillin–streptomycin mixture (contains 10,000 IU/ml potassium penicillin and 10,000 µg/ml of streptomycin sulfate), Dulbecco’s modified eagle’s medium (DMEM), fetal bovine serum (FBS), Hanks’ balanced salt solution (HBSS) (referred to as ‘standard buffer’ when pH adjusted to 7.4), Phosphate Buffered Saline (PBS; 1× and 10×), and Trypan blue solution (0.4%) were purchased from Lonza Verviers SPRL (Verviers, Belgium). ITS +™ Premix (contains insulin 6.25 mg/l, transferrin 6.25 mg/l, selenious acid 6.25 mg/l, bovine serum albumin 1.25 g/l and linoleic acid, 5.35 mg/l) was purchased from BD Biosciences (Erembodegem, Belgium). Sulfuric acid (95–97%) was purchased from Chem-Lab NV (Zedelgem, Belgium). All BAs, collagenase type IV (from Clostridium histolyticum), ECM gel (from Engelbreth-Holm-Swarm murine sarcoma), recombinant human insulin, dexamethasone, urea, diacyl monoxime, thiosemicarbazide, iron (III) chloride hexahydrate, ortho-phosphoric acid, ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA), Dulbecco’s modified eagle’s medium 10× (DMEM 10×), and 5(6)-carboxy-2′,7′-dichlorofluorescein diacetate (CFDA) were purchased from Sigma-Aldrich (Schnelldorf, Germany). Cyclosporin A, troleandomycin, and bosentan were purchased from Sequoia Research Products Ltd, UK. HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) was purchased from MP Biochemical (Illkirch, France). 48 and 24-well sterile cell culture plates were purchased from Greiner Bio-One BVBA (Wemmel, Belgium). Thermostat 96-well plates (for assay) were kindly provided by Greiner Bio-One BVBA (Wemmel, Belgium).
Isolation and culture of rat hepatocytes in sandwich configuration. Hepatocytes were isolated from male Wistar rats (170–200 g) based on a two-step collagenase perfusion method, as described previously, with minor modifications (Chatterjee et al., in press). Briefly, 24-well plates were coated with ice-cold neutralized collagen solution, and placed overnight at 37 °C in a humidified incubator, and hydrated with PBS before use. Hepatocytes were seeded at a density of 0.5 × 10⁶ cells/well, in 500 μl well of day-0 medium. After incubating the cells at 37 °C in a humidified atmosphere with 5% CO₂ (Binder CO₂ incubator, Binder GmbH) for 1–2 h, unattached cells were removed by shaking the plate and immediately aspirating the medium. To obtain a “sandwich” configuration, the cells were overlaid with 50 μl of rat tail collagen solution (1.5 mg/ml, pH 7.4) (day-0). One hour later, pre-warmed day-0 medium was added onto the cultures which were kept in a humidified atmosphere with 5% CO₂. The medium was changed every day with culture medium. The capacity of the hepatocytes to convert ammonia to urea was used to assess the overall biochemical function and integrity of rat and human SCH. Urea formation in rat and human SCH. The capacity of the hepatocytes to convert ammonia to urea was used to assess the overall biochemical function and integrity of rat and human SCH. Urea formation in rat and human SCH. Urea formation in rat and human SCH.

Table 1
The demographics and batch characteristics of the cryopreserved human hepatocyte batches (donors) used in this study. * indicates that batch SC1034 was a freshly plated human hepatocyte batch. " indicates that batch SC1034 was a freshly plated human hepatocyte batch. Bile canalicil formation was evaluated (see Supplementary Fig. 1) based on CDF excretion capacity of the SCHH, with fluorescence microscopy. The symbols for the human hepatocyte batches used in Figs. 3A and B are also represented.

<table>
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<th>Lot number (symbol)</th>
<th>Gender</th>
<th>Race</th>
<th>Age (years)</th>
<th>Viability [%]</th>
<th>Yield (million/vial)</th>
<th>Bile canalicil function</th>
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<td>S240908 (●)</td>
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<td>S2203LT (●)</td>
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<td>(NA)</td>
<td>(NA)</td>
<td>12</td>
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</tr>
</tbody>
</table>

NA = not available.
diacetate (CDDDFA) in standard buffer. After 10 min incubation at 37 °C, the buffer was removed, hepatocytes were washed twice with standard buffer before fresh buffer was added. Hepatocytes and bile networks were imaged (both by fluorescence and light microscopy) with a VisiCam® 3.0 camera (VWR International, Leuven, Belgium), mounted on an Olympus IX70 inverted tissue culture microscope (Olympus Optical Co., GMBH, Hamburg, Germany). A monochromator (Polychrome IV; Till Photonics, Oberhausen, Germany) was used to generate the excitation wavelength (490 nm). For fluorescence microscopy a U-MWIB3 mirror unit was used (emission filter: 510 nm (long pass); dichroic mirror: 505 nm).

**Incubations with BAs.** Forty to sixty-fold concentrated solutions of a BA mixture (referred to as 40× and 60× BA mixture) consisting of the five quantitatively most important BAs present in the human plasma, were used (Gnewuch et al., 2009; Scherer et al., 2009; Xiang et al., 2010). The 40× BA mixture consisted of 52.8 μM glycodeoxycholic acid (GCDCA), 15.6 μM of chenodeoxycholic acid (CDCA), 15.2 μM of glycocholic acid (GCA), 16 μM of deoxycholic acid (DCA), and 14 μM of glycodeoxycholic acid (GCDCA); the 60× BA mix consisted of 79.2, 23.4, 22.8, 24 and 21 μM of GCDCA, CDCA, GCA, DCA and GCA, respectively. Hepatocytes were first incubated with the test compounds alone for 2 h, to provide the test compound time to interfere with bile acid transporters. Subsequently, the test compound and the 40–60× BA mixtures (i.e. 60× for experiments with SCHH and 40× for experiments with SCHH) were co-incubated for 22 h. After the incubations, urea assay was performed for quantitative assessment of (compromised) hepatocytes functionality. For SCHH from two donors, hepatocytes were re-exposed a second time for 24 h to compound and BA mixture, followed by another urea assay, to assess the effect of co-exposure on in vitro cholestasis potency. All the BA solutions were prepared in day-1 medium.

**Data analysis.** The E_{max} model was used to describe the concentration-dependency of the inhibitory effect of troglitazone, bosentan and chlorpromazine (Fig. 1) with and without BA mixture, on the capacity of hepatocytes to produce urea:

\[
E = E_{max} - \left( E_{max} - E_0 \right) \frac{C^n}{C_{n+1} + IC_{50}} \tag{1}
\]

where E is the urea production by hepatocytes, E_{max} is the urea production under control condition (no compound was added), E_0 is the urea production at the maximum inhibitory effect of compound (and BA mixture), E_{max} − E_0 is the maximum inhibitory effect. The IC_{50} is the compound concentration (with or without BA mixture) causing 50% inhibition of urea formation. The parameter “n” denotes the Hill factor. The best fits of the above equation to the individual urea formation data sets were obtained by non-linear regression analysis with the NLS package in R version 2.15.1. The inverse of the experimentally obtained standard deviations were used for weighing.

To quantify the ability of a test compound to exert toxicity by disturbing BA homeostasis in vitro, a drug-induced cholestasis index (DICI) was calculated as follows:

\[
DICI = \frac{Urea \text{ formation}_{\text{test compound} - \text{bile acids}} - Urea \text{ formation}_{\text{test compound alone}}}{Urea \text{ formation}_{\text{test compound alone}}} \tag{2}
\]

DICI values were calculated for each compound at every concentration examined. Compounds were classified empirically based on their DICI values: (i) compounds with mild or no potential to cause cholestasis: DICI > 0.8; (ii) compounds with moderate cholestasis risk: 0.8 ≥ DICI > 0.5 and (iii) compounds with high risk to cause cholestasis: DICI ≤ 0.5.

Safety margins were calculated for each compound, based on the C_{max} (μM, mean peak plasma concentration in human, obtained from clinical reports) and the lowest in vitro concentration (μM) yielding a DICI < 0.8, as follows:

\[
\text{Safety Margin(SM)} = \frac{\text{Lowest concentration(μM) yielding DICI} \leq 0.8}{C_{\max}(μM)} \tag{3}
\]

When DICI was >0.8 for the highest concentration tested, a minimum safety margin was reported (SM > highest concentration/C_{max} (μM)). For the test compounds evaluated, the concentration range included between the C_{max} and C_{max}/10 was considered to be the therapeutic plasma concentration range (see Figs. 2 and 3).

**Statistics.** For troglitazone (Fig. 1), the ANOVA (F-test) in MS Excel version 2007 was used to evaluate statistical significance of differences between urea formation profiles by SCHH treated with troglitazone alone and with troglitazone plus the 60× BA mixture: separately fit profiles for troglitazone alone or for troglitazone plus BAs were compared to the simultaneous fit obtained with both data sets combined. For each concentration of chlorpromazine and bosentan (Fig. 1), a two-tailed student t-test was used to assess the statistical significance of the differences in urea formation (nmol/well) between SCHH treated with compounds alone or SCHH treated with compound plus 60× BA mixture. The criterion for statistical significance was p < 0.05.

**Results**

**Influence of BAs on concentration-dependent toxicity of bosentan, troglitazone and chlorpromazine in SCHH: proof of concept**

The working hypothesis of the present study was that SCHH would be sensitized to the cytotoxic effects of BAs upon co-incubation with compounds reported to cause cholestasis in the clinic. To test this hypothesis, SCHH were exposed to increasing concentrations of bosentan, chlorpromazine and troglitazone with or without a 60× BA mixture. For bosentan concentrations > 100 μM, SCHH became sensitive to the toxic effects of BAs. This was reflected by decreased urea formation in SCHH co-incubated with bosentan and BA mixture as compared to bosentan or BA mixture alone (Fig. 1A).

Similarly, SCHH were also sensitized to the cytotoxic effects of BAs when co-incubating the cholestatic compounds chlorpromazine and troglitazone with the 60× BA mixture (Figs. 1B–C). In case of chlorpromazine, the difference in urea formation between SCHH treated with chlorpromazine alone and SCHH co-incubated with chlorpromazine and BA mixture was only observed at the highest concentration (30 μM) of chlorpromazine treatment (Fig. 1B). However, for troglitazone, co-incubation with the BA mixture resulted in decreased urea formation for all concentrations tested (except for 50 μM). The concentration-dependent urea formation profiles for troglitazone with and without 60× BA mixture were significantly different from each other (p < 0.05).

The effect of BAs in modulating in vitro cytotoxicity of the cholestatic compounds is further illustrated in Table 2. The IC_{50} for reduction of DICI values by SCHH treated with troglitazone and chlorpromazine in SCHH: proof of concept was only observed at the highest concentration (30 μM) yielding a DICI ≤ 0.8, as follows:

\[
\text{Safety Margin(SM)} = \frac{\text{Lowest concentration(μM) yielding DICI} \leq 0.8}{C_{\max}(μM)} \tag{3}
\]

When DICI was >0.8 for the highest concentration tested, a minimum safety margin was reported (SM > highest concentration/C_{max} (μM)). For the test compounds evaluated, the concentration range included between the C_{max} and C_{max}/10 was considered to be the therapeutic plasma concentration range (see Figs. 2 and 3).

The effect of co-incubation with a BA mixture on toxicity of known cholestatic compounds in different batches of SCHH

To determine the effect of the 60× BA mixture on the concentration-dependent toxicity of known cholestatic compounds in multiple batches of SCHH, cyclosporin A (n = 6 batches), troglitazone (n = 3), bosentan (n = 1), chlorpromazine (n = 2), ritonavir (n = 2) and glyburide (n = 3), were incubated with and without BA mixture.
(Fig. 2). Mean (± SEM) DICI values (Eq. (2)) were determined for each compound following the urea formation measurements in three wells with and three wells without BAs. DICI values obtained from incubations with cyclosporin A at ≥ 10 μM and for troglitazone at ≥ 75 μM in different batches of SCRH were lower than 0.8. For bosentan, chlorpromazine and glyburide, an increasing number of DICI values were found to be lower than 0.8, as the concentration of the incubated compounds increased. In contrast, DICI values remained > 0.8 for ritonavir at all the concentrations tested (1–200 μM).

For each compound a safety margin (SM) was obtained (Eq. (3)) based on the ratio of lowest in vitro concentration for which DICI ≤ 0.8, to the reported plasma C\textsubscript{max}. SM values ranged between 5.6 for cyclosporin A to 244 for glyburide. For ritonavir a SM > 13.1 was reported since the highest evaluated in vitro concentration yielded a DICI > 0.8 (Table 3).

Effect of co-incubation with a BA mixture on toxicity of known cholestatic compounds in different batches of SCHH

The mean (± SEM) DICI values shown in Figs. 3A–B were determined following incubations with and without 40× BA mixture in SCHH obtained from various donors (demographics in Table 1) at different concentrations of cyclosporin A (n = 6 batches), troglitazone (n = 4), bosentan (n = 4), chlorpromazine (n = 2), ritonavir (n = 2), ticlopidine (n = 1), midecamycin (n = 3), rosiglitazone (n = 1), erythromycin estolate (n = 1) and troleandomycin (n = 2). Consistent with the results in SCRH, experiments with cyclosporin A at ≥ 10 μM and troglitazone ≥ 75 μM produced DICI values below 0.8. Incubations with 1 μM of bosentan in one out of four SCHH batches (lot number S240908) yielded a DICI lower than 0.8. For chlorpromazine, midecamycin, ticlopidine (100 μM), ritonavir, DICI values were observed to go below 0.8 at
more than one concentration. However, for rosiglitazone, erythromycin estolate and troleandomycin, none of the DICI values were $\leq 0.8$.

Similar to SCRH, a SM value was calculated for compounds in SCHH. SM values varied between 0.2 in case of bosentan to 15.6 for troglitazone (Table 3). Compounds with SM $b 30$ are considered to show significant risk to cause cholestasis in the clinic (see Discussion section for details).

Effect of co-incubation with a BA mixture on toxicity of negative control compounds in different batches of SCRH and SCHH

Compounds that mediate hepatotoxicity by mechanisms other than interfering with BA homeostasis, along with a known non-hepatotoxicant (warfarin) were selected as negative control compounds. Diclofenac and valproic acid were used as negative control compounds in SCRH (Fig. 4), yielding DICI values $N 0.9$ for all the concentrations tested. In SCHH, mean ($\pm$ SEM) DICI values determined for 20 $\mu$M amiodarone, 1 mM acetaminophen and 500 $\mu$M warfarin were always $>0.90$ (Table 4).

Effect of repeated exposure of SCHH to compound and BA mixture on in vitro estimation of cholestasis potential

The influence of repeated exposure to compounds and the 40 $\times$ BA mixture on the toxicity of bosentan, midecamycin, ritonavir and troleandomycin was evaluated in two batches of SCHH (Table 5). Following the urea assay after 24 h incubation, the same cells were re-exposed to the same concentration of compound and BA mixture for another 24 h. The DICI values were determined both after 24 and 48 h of co-incubation. Consistently lower DICI values were obtained after 48 h co-incubation compared to 24 h co-incubation. For ritonavir a DICI $\leq 0.8$ was obtained after re-exposure (2 incubation periods of 24 h, Table 5), which contrasted to the unchanged DICI following single exposure.

Correlation between safety margin in SCHH and clinical incidences of cholestasis

To correlate the in vitro estimated cholestasis potential of known cholestatic compounds in the current assay (in SCHH) with their
clinically-reported incidence data for cholestasis, safety margins were calculated (Eq. (3)). The incidences of hepatotoxicity were obtained from literature (Dhillon and Keating, 2009; Humbert et al., 2007; Klintmalm et al., 1981; Larrey and Erlinger, 1988; Naschitz et al., 1995; Sulkowski, 2004; Ticktin and Zimmerman, 1962). As no literature incidence data were available for midecamycin, it was not included in this correlation analysis. For troleandomycin and ritonavir DICI values obtained after 24 h exposure remained >0.8 at all concentrations, therefore minimum SM values were used for plotting. Two separate correlation analyses were carried out (one with and one without bosentan): bosentan yielded a marginally decreased DICI value (≤0.8, but standard deviation overlapping with 0.8 cut-off) for a low concentration only (no concentration-dependent decrease in DICI). Compounds yielding a safety margin > 30 (rosiglitazone, erythromycin estolate) were not included in the analysis. For cyclosporin A and bosentan, the reports from two independent literature sources were combined (weighed to the number of patients in the literature reports), while for ritonavir a range was obtained in the literature (the mean of the range was used for correlation, Table 6). No particular clinical marker for cholestasis was selected, as the individual reports employed different markers of general hepatotoxicity. Fig. 5 illustrates that clinical incidence of cholestasis decrease with increasing calculated safety margin for cyclosporin A, bosentan, troglitazone, ritonavir, chlorpromazine and ticlopidin. A linear correlation ($r^2 = 0.85$; $r^2 = 0.86$, when bosentan included) was obtained between safety margin and incidence (%) of cholestasis.

**Discussion**

At the hepatic level, xenobiotics can disturb BA homeostasis, by interfering with any of the following stages of BA disposition: (i) uptake of BAs from the basolateral side of the hepatocytes, (ii) de-novo synthesis of the BAs (iii) metabolism/conjugation inside the hepatocytes, (iv) efflux of BAs to the bile canaliculi via canalicular transporters, and (v) sinusoidal efflux of BAs via basolateral efflux transporters. Consequently, a holistic in vitro model that covers the various stages of hepatic BA disposition would be best equipped to accurately predict cholestatic potential of a drug (or drug candidate) altering intrahepatic

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**Fig. 3.** A–B: DICI values (at various concentrations) and SM values obtained in SCHH with cyclosporin A, troglitazone, bosentan, chlorpromazine, ritonavir, ticlopidine, midecamycin, rosiglitazone, erythromycin estolate and troleandomycin. Points represent mean (± SEM) DICI values obtained with human hepatocytes from different donors (symbols refer to unique donors across different panels; see Table 1 for donor information). DICI values were calculated based on the urea assay following 24 h co-incubation of the compounds with or without BA mixture (three wells with and three wells without BA mix), as described in the Material and methods section. A DICI value of 0.8 is indicated by the dotted line on the Y-axis, while the dotted line on the X-axis represents the reported Cmax for each compound. The shaded area covers DICI values below 0.8 (i.e. flagged for cholestasis risk) for concentrations within the therapeutic plasma concentration range (between Cmax/10 and Cmax).

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BA homeostasis. Hepatocytes in sandwich-culture have been shown to preserve the functions of proteins controlling the different stages of BA disposition (Chatterjee et al., in press; De Bruyn et al., 2013). We have presently developed a SCH based assay that can classify drugs/compounds based on their potential to cause cholestasis in the clinic via altered BA homeostasis. In vitro biliary excretory capacity of the SCH used in the present study was qualitatively verified at day-5 by measuring MRP2-mediated CDF efflux into bile canaliculi (as an in-process control, Supplementary Fig. 1).

For the purpose of the present study we have composed a BA mixture, containing five BAs based on their quantitative importance in human plasma, as reported previously (Gnewuch et al., 2009; Scherer et al., 2009; Xiang et al., 2010). The toxicity exerted by the compounds (alone or in combination with the BA mixture) was measured by the urea assay, which is a marker for integrity of liver specific-function (Chatterjee et al., in press). The 40×–60× concentrated BA mixtures selected for the present study are higher than the physiological BA levels in plasma, but become relevant in case of cholestasis. Hepatic BA concentrations have been reported to reach 430–800 μM in case of cholestasis (Fischer et al., 1996; Rolo et al., 2003).

The concentrations of the BA mixture were selected such that they would not affect the urea formation by the cultures when incubated alone. In contrast, co-incubation of the same cultures in the presence of the BA mixture and increasing concentrations of a cholestatic compound should lead to toxicity (decreased urea formation). In other words, it was hypothesized that the presence of BAs in the extracellular medium would sensitize the hepatocytes towards the cholestatic action of the compounds interfering with BA homeostasis. An increased toxicity in the presence of the BA mixture with 100–200 μM of bosentan, 30 μM of chlorpromazine and with 75–150 μM of troglitazone, clearly vindicates this hypothesis (Fig. 1). This suggests that at those concentrations, the hepatocytes showed decreased ability to dispose of the added BAs, consistent with the cholestatic action of the compounds.

Based on the design of this new in vitro model as well as the results obtained, we introduce the concept: “drug-induced cholestasis index” (DICI). DICI is a relative measure for the residual urea formation when a cholestatic drug is incubated in the presence of BAs as compared to the urea formation when the drug is applied separately. DICI values classify compounds according to their ability to potentiate the in vitro cytotoxicity of the BAs in hepatocytes, and this is expected to be related to the potential of compounds to cause cholestasis in vivo. The DICI value measurements in human hepatocytes are particularly relevant for clinical conditions, as they may provide indication of the cholestatic signature of the compound in the clinic. Variability between the different batches of human hepatocytes regarding the measured increase in

Table 2

<table>
<thead>
<tr>
<th>Compound name</th>
<th>SCRH</th>
<th>SCHH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bosentan</td>
<td>13.5</td>
<td>0.14</td>
</tr>
<tr>
<td>Ritonavir</td>
<td>&gt;13.1</td>
<td>&gt;6.60</td>
</tr>
<tr>
<td>Cyclosporin A</td>
<td>5.60</td>
<td>8.30</td>
</tr>
<tr>
<td>Midecamycin</td>
<td>(ND)</td>
<td>10.2</td>
</tr>
<tr>
<td>Troleandomycin</td>
<td>(ND)</td>
<td>&gt;10.2</td>
</tr>
<tr>
<td>Chlorpromazine</td>
<td>31.9</td>
<td>10.6</td>
</tr>
<tr>
<td>Ticlopidine</td>
<td>(ND)</td>
<td>12.4</td>
</tr>
<tr>
<td>Troglitazone</td>
<td>11.7</td>
<td>15.6</td>
</tr>
<tr>
<td>Erythromycin estolate</td>
<td>(ND)</td>
<td>&gt;68.7</td>
</tr>
<tr>
<td>Rosiglitazone</td>
<td>(ND)</td>
<td>&gt;96.2</td>
</tr>
<tr>
<td>Glyburide</td>
<td>244</td>
<td>(ND)</td>
</tr>
</tbody>
</table>

ND = not determined.

Table 3

<table>
<thead>
<tr>
<th>Compound name</th>
<th>SCRH</th>
<th>SCHH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bosentan</td>
<td>135 ± 5.5</td>
<td>&gt;200</td>
</tr>
<tr>
<td>Chlorpromazine</td>
<td>250 ± 18</td>
<td>&gt;30</td>
</tr>
<tr>
<td>Troglitazone</td>
<td>923 ± 38</td>
<td>126 ± 0.2</td>
</tr>
</tbody>
</table>
toxicity of compounds in the presence of BAs may reflect to some extent the variability in toxic response of these compounds in the clinic. For instance, a DICI of 0.66 ± 0.08 was obtained with 20 μM cyclosporin A in batch S0906A, while in S240908 a DICI of 0.21 ± 0.12 was obtained with 15 μM of cyclosporin A treatment. A similar interbatch variability was noted for troglitazone, ritonavir, bosentan among other compounds. These findings support the use of multiple batches of SCHH to achieve adequate sensitivity for detecting cholestatic drug candidates.

Cyclosporin A and troglitazone are two known cholestatic compounds that elicited a clear concentration-dependent decrease of DICI in different batches of SCRH and SCHH (Figs. 2 and 3). In addition, the concentration-dependent decrease of DICI was also observed for bosentan, chlorpromazine and troglitazone in rat hepatocytes (Fig. 1, Supplementary Table 1). The concentration dependency of the DICI values strengthens the hypothesis that disturbances in BA homeostasis mediated by the compounds, contributes to the cholestatic effect.

Rosiglitazone is an analog of troglitazone with a decreased risk of hepatotoxicity. With troglitazone treatment, 1.9% of the patients had ALT > 3 times upper limit of normal (ULN), as opposed to only 0.17% patients with rosiglitazone (Lebovitz et al., 2002). However, previous reports suggested a strong BSEP inhibition potential of rosiglitazone (Dawson et al., 2012). When troglitazone and rosiglitazone were evaluated for cholestasis potential with our assay in the same batch of human hepatocytes, troglitazone yielded a DICI ~ 0, while the DICI value for rosiglitazone was 1.2 at the same concentration (100 μM). The higher DICI for rosiglitazone compared to troglitazone illustrates that the assay is competent to differentiate between cholestatic and non-cholestatic compounds with similar chemical motifs.

DICI values in the range 1.5–2.0 were obtained for glyburide and ritonavir in SCRH. These compounds are reported to interfere with the BA uptake transporters (Leslie et al., 2007). At high concentrations, these compounds might prevent the BAs to be taken up by the hepatocytes, thus decreasing the intracellular accumulation of BAs and in turn displaying a protective effect as illustrated by DICI values exceeding 1. These findings suggest further investigation regarding a sequential incubation design to minimize direct interference between BAs and cholestatic compounds at the hepatic uptake level.

Further to DICI, corresponding safety margin (SM) values were calculated. The SM reflects the ratio of the lowest concentration of the compound that yields a DICI ≤ 0.8 to the mean peak plasma concentration that the compound (drug) reaches in the clinic (Cmax). To account for different sensitivity and/or distinct intracellular accumulation and metabolism as applicable in vitro versus in vivo, a SM cut-off value of 30 was used to classify compounds as cholestatic versus non-cholestatic. Thus a compound with a SM < 30 is considered to show significant cholestasis risk in the clinic. A SM cut-off value of 30 has been suggested previously for predicting clinical toxicity via in vitro models, e.g. for cardiotoxicity (Yao et al., 2008). The rationale for employing this safety margin towards compound decisions can be further illustrated by the case of cyclosporin A, which has been reported to accumulate in the liver several folds more than in the plasma (Lacerda et al., 1995). Consistently, when the pharmacokinetic profile of cyclosporin A was simulated in SimCYP (Version 12, release 1, Sheffield, UK), the hepatic Cmax was found to be 7.6 times higher than plasma Cmax (Supplementary Fig. 2). While hepatocyte accumulation of cyclosporin A is likely to also occur in SCH, the actual accumulation ratio may be different due to distinct transporter and metabolizing enzyme expression profiles between in vitro and in vivo. Such in vitro–in vivo discrepancies necessitate the use of a safety margin to support reliable compound decisions. In addition, recent reports (Anthérieu et al., 2013; Dawson et al., 2012) suggest that hepatotoxicity manifested by a compound is contributed by different toxicity-pathways acting simultaneously. Again, the safety margin will help to compensate for in vitro–in vivo discrepancies in the quantitative role of these pathways.

For troglitazone, chlorpromazine and ticlopidine, multiple mechanisms have been reported to contribute to the toxicity profiles. Reactive metabolite formation has been described for troglitazone along with BSEP inhibition (He et al., 2004). In addition, the metabolite troglitazone-sulfate has been reported to be a much stronger inhibitor of BSEP compared to the parent compound (Funk et al., 2001).

Table 4
<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration (μM)</th>
<th>Lot number</th>
<th>DICI ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetaminophen</td>
<td>1000</td>
<td>S1109YT</td>
<td>0.93 ± 0.34</td>
</tr>
<tr>
<td>Amiodarone</td>
<td>20</td>
<td>SC1034</td>
<td>1.24 ± 0.05</td>
</tr>
<tr>
<td>Warfarin</td>
<td>500</td>
<td>S0212A</td>
<td>1.31 ± 0.19</td>
</tr>
</tbody>
</table>

Table 5
Effect of repeated exposure of SCHH to compound and BA mixture on drug-induced cholestasis index (DICI) values (mean ± SD). Cyclosporin A and troglitazone were evaluated in the same batch (S2203LT), while other compounds in a different batch (S0312VT). The DICI values ≤ 0.80 are in bold.

<table>
<thead>
<tr>
<th>Compound</th>
<th>DICI (± SEM)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 h co-incubation</td>
<td>48 h co-incubation</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bosentan 1 μM</td>
<td>1.11 ± 0.09</td>
<td>1.02 ± 0.13</td>
<td></td>
</tr>
<tr>
<td>Bosentan 5 μM</td>
<td>0.98 ± 0.03</td>
<td>0.85 ± 0.07</td>
<td></td>
</tr>
<tr>
<td>Bosentan 10 μM</td>
<td>1.03 ± 0.09</td>
<td>0.98 ± 0.11</td>
<td></td>
</tr>
<tr>
<td>Bosentan 200 μM</td>
<td>0.85 ± 0.07</td>
<td>0.75 ± 0.16</td>
<td></td>
</tr>
<tr>
<td>Cyclosporin A 15 μM</td>
<td>0.74 ± 0.15</td>
<td>0.46 ± 0.18</td>
<td></td>
</tr>
<tr>
<td>Midecamycin 100 μM</td>
<td>1.00 ± 0.19</td>
<td>1.07 ± 0.07</td>
<td></td>
</tr>
<tr>
<td>Ritonavir 1 μM</td>
<td>0.95 ± 0.09</td>
<td>0.92 ± 0.12</td>
<td></td>
</tr>
<tr>
<td>Ritonavir 50 μM</td>
<td>1.02 ± 0.12</td>
<td>0.92 ± 0.08</td>
<td></td>
</tr>
<tr>
<td>Ritonavir 100 μM</td>
<td>0.99 ± 0.08</td>
<td>0.80 ± 0.39</td>
<td></td>
</tr>
<tr>
<td>Troglitazone 100 μM</td>
<td>0.66 ± 0.13</td>
<td>0.61 ± 0.09</td>
<td></td>
</tr>
<tr>
<td>Troglitazone 10 μM</td>
<td>1.00 ± 0.09</td>
<td>0.83 ± 0.26</td>
<td></td>
</tr>
<tr>
<td>Troglitazone 20 μM</td>
<td>1.12 ± 0.13</td>
<td>0.91 ± 0.08</td>
<td></td>
</tr>
</tbody>
</table>
The vesicles/BSEP over-expressed cell lines lack the metabolic machineries required to produce the clinically relevant metabolites, including bosentan (●), which yielded borderline DICI values (±0.8) two times in SCHH (see Discussion section). Incidences were obtained from clinical reports of hepatotoxicity of these drugs. The mean incidence from two different reports was used for both cyclosporin A and bosentan (weighted for the number of patients). For chlorpromazine and ritonavir the reported range of cholestasis incidence is shown. Compounds with a SM > 30 were not included in the analysis (see Discussion section). Troleandomycin and ritonavir (●) did not yield DICI values ≤ 0.8, however the minimum SM values based on the highest concentration tested in vitro were below 30. For midecamycin no literature reports of incidence of cholestasis (%) were available, hence it was not included in this correlation analysis.

Fig. 5. Correlation between literature reports of incidences of cholestasis (%) and SM values (r² = 0.85) in SCHH, for cyclosporin A, troglitazone, chlorpromazine, ticlopidine and troleandomycin. The dotted line represents the correlation (r² = 0.86) including bosentan (●), which yielded borderline DICI values (<0.8) two times in SCHH (see Discussion section). Incidences were obtained from clinical reports of hepatotoxicity of these drugs. The mean incidence from two different reports was used for both cyclosporin A and bosentan (weighted for the number of patients). For chlorpromazine and ritonavir the reported range of cholestasis incidence is shown. Compounds with a SM > 30 were not included in the analysis (see Discussion section). Troleandomycin and ritonavir (●) did not yield DICI values ≤ 0.8, however the minimum SM values based on the highest concentration tested in vitro were below 30. For midecamycin no literature reports of incidence of cholestasis (%) were available, hence it was not included in this correlation analysis.

Regarding the correlation shown in Fig. 5 as well as the classification represented in Fig. 6, the in vitro data were unambiguous for the majority of the test compounds. However, further investigation is warranted for bosentan, ritonavir, troleandomycin, and erythromycin estolate. The latter represents the only misclassified compound by our in vitro assay. In SCHH, the SM for erythromycin was > 69, while the DICI value appeared to increase with increasing concentration. This again suggests possible interference between BAs and erythromycin at the level of hepatic uptake, as also mentioned above for glyburide and ritonavir in rat hepatocytes. Moreover, a higher inhibitory effect on the uptake of BAs has been suggested for erythromycin estolate in SCHH, which will further decrease the accumulation and hence the expected 

Table 6

<table>
<thead>
<tr>
<th>Drug</th>
<th>hBSEP inhibition</th>
<th>Incidence (%)</th>
<th>Clinical markers</th>
<th>Patient population</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bosentan</td>
<td>38.1</td>
<td>7.2</td>
<td>ALT &gt; 3 ULN</td>
<td>4623 pulmonary arterial hypertension patients</td>
<td>Dawson et al. (2012), Humbert et al. (2007)</td>
</tr>
<tr>
<td>Chlorpromazine</td>
<td>147.6</td>
<td>2–5</td>
<td>ALT &gt; 3 ULN</td>
<td>658 pulmonary arterial hypertension patients</td>
<td>Dawson et al. (2012), Larrey and Erlinger (1988)</td>
</tr>
<tr>
<td>Cyclosporin A</td>
<td>0.5</td>
<td>2–5</td>
<td>ALT &gt; 3 ULN</td>
<td>66 renal transplant patients</td>
<td>Dawson et al. (2012), Klintmalm et al. (1981)</td>
</tr>
<tr>
<td>Erythromycin estolate</td>
<td>4.1</td>
<td>2</td>
<td>ALP &gt; 3 ULN</td>
<td>705 kidney transplant, 112 heart transplant, 75 liver transplant</td>
<td>Dawson et al. (2012), Klintmalm et al. (1981)</td>
</tr>
<tr>
<td>Ritonavir</td>
<td>2.2</td>
<td>5.3–9.5</td>
<td>ALT, AST &gt; 3 ULN</td>
<td>1270 infected patients</td>
<td>Dawson et al. (2012), Lewis and Zimmerman (1999)</td>
</tr>
<tr>
<td>Rosiglitazone</td>
<td>6.4</td>
<td>0.17</td>
<td>ALP &gt; 3 ULN</td>
<td>3503 diabetic patients</td>
<td>Morgan et al. (2010), Sulkowski (2004)</td>
</tr>
<tr>
<td>Ticlopidine</td>
<td>74</td>
<td>4</td>
<td>ALP &gt; 3 ULN</td>
<td>50</td>
<td>Dawson et al. (2012), Web reference 2</td>
</tr>
<tr>
<td>Troglitazone</td>
<td>2.7</td>
<td>1.9</td>
<td>ALP &gt; 3 ULN</td>
<td>2510 diabetic patients</td>
<td>Dawson et al. (2012), Lebovitz et al. (2002)</td>
</tr>
<tr>
<td>Troleandomycin</td>
<td>(ND)</td>
<td>4</td>
<td>ALP &gt; 3 ULN</td>
<td>50</td>
<td>Dhillon and Keating (2009)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a Alanine aminotransferase.
b Upper limit of normal.
c Alkaline phosphatase.
d Upper limit of reference range.
increased toxicity in the presence of BAs may not be evident (Ansedo et al., 2010). In addition, erythromycin has been shown to accumulate in the liver; 150-fold higher hepatic concentrations than serum concentrations have been reported for erythromycin in rats (Lee et al., 1953). Although the uptake transporters are qualitatively maintained in SCH, a down-regulation of uptake transporters with culture time has been reported (Tchaparian et al., 2011). This may decrease the uptake of the drug and hence intracellular accumulation compared to the in vivo situation. Investigation with higher concentrations of erythromycin can be suggested to tackle the issue.

Ritonavir did not yield a DICI ≤ 0.8 after 24 h co-incubation, with the current assay design. This resulted in a “minimum” SM (≥6.6), meaning that this HIV protease inhibitor could not be unambiguously classified as safe (SM cut-off 30) based on the present in vitro data. Consequently, the “minimum” SM was used to correlate to incidence (%) of hepatotoxicity in Fig. 5. The higher hepatotoxicity incidences with ritonavir may be attributed to the fact that the patients treated with ritonavir have already become susceptible to liver injury due to the existing HIV infection. In addition to that, ritonavir is often co-administered with other anti-HIV drugs such as azathipron which potentiates the cholestasis incidences in the clinic (Rakotondravelo et al., 2012). The safety margin calculated here is obtained with human hepatocytes that are not infected with HIV, neither exposed to other drugs. A “minimum” SM was also employed for the antibiotic troleandomycin (SM > 10 in SCHH), which could thus also not be classified as non-cholestatic. This is consistent with reports of cholestasis jaundice (Tickt and Zimmerman, 1962) warranting further investigation with a higher concentration and possibly different incubation design.

Although bosentan did show a DICI ≤ 0.8 at 1 μM in SCHH (DICI = 0.75 ± 0.14), there was no evidence of a concentration-dependent increase in cholestasis potential. In contrast, bosentan did show concentration-dependent increase in cholestatic potential in SCRH (Figs. 1 and 2). In addition, following re-exposure of SCHH to 200 μM bosentan and BA mixture, the DICI value obtained was ≤0.8 (DICI = 0.75 ± 0.16). Taken together this in vitro assay classifies bosentan as a compound with a potential cholestatic risk, but requiring further investigation (Figs. 5 and 6). Our findings illustrate the differential and complex interaction of bosentan with rat and human transporters responsible for BA disposition. It is noteworthy that bosentan has more potent interaction with rat Ntcp compared to human NTCP (Leslie et al., 2007).

Repeated exposure to compounds and BA mixture was examined to improve the sensitivity of the in vitro cholestasis model (Table 5). The non-destructive nature of urea assay provided the opportunity to re-expose the cells with compound and BA mixture, after one urea assay has been carried out. As mentioned above, 100 μM ritonavir produced a DICI ≤ 0.8 after repeated (but not single) exposure. Also for cyclosporin A, troleandomycin, bosentan, and treloandomycin, DICI values decrease after repeated exposure.

The negative control compounds were selected such that they are known to cause hepatotoxicity (diclofenac, valproic acid, amiodarone, acetaminophen), but by mechanisms other than interaction with BA homeostasis, along with the non-hepatotoxic warfarin. None of the negative control compounds produced a DICI value ≤ 0.8 in both SCHH and SCRH. Diclofenac, acetaminophen and amiodarone have been reported to cause hepatotoxicity by reactive metabolite formation while for valproic acid, the intrinsic toxicity coupled with a reactive acyl glucuronide moiety have been implicated in its hepatotoxicity (Bort et al., 1999; Kiang et al., 2011; Manyike et al., 2000; Zahno et al., 2011). The test results with negative control compounds suggest that the current assay will not result in increased in vitro toxicity of compounds in the presence of BA mixture, if the compounds do not interfere with BA disposition in vivo.

Our data warn for ignoring species differences in drug toxicity. The previously reported in vivo toxicity of chlorpromazine and troglitazone in rat could not explain the observed clinical toxicity. For chlorpromazine, only moderate increase (≤2 times) of aspartate amino transferase (AST) and bilirubin levels have been reported in rat (Schoonen et al., 2007). Another study reported no significant increase in the levels of bilirubin or ALP, however a three times increase of γ-glutamyl transpeptidase was reported (Obata, 1983). In addition, in the in vitro BSEP/Bsep inhibition studies could not distinguish between rat and human regarding the different sensitivity to the hepatotoxic effects of chlorpromazine. Similar hBSEP and rBsep inhibition IC50 values (147 μM in human versus 122 μM in rat) were reported for chlorpromazine (Dawson et al., 2012). However, in the current model a > 3 times higher SM was obtained for chlorpromazine in rat compared to human, thus corroborating the species-specific cholestatic/hepatotoxic response observed. Also the animal models with currently available biomarkers could not detect the cholestatic/hepatotoxic nature of troglitazone in rat (Li et al., 2002; Marra et al., 2005), although strong Bsep/Bsep inhibition was observed in vitro (Dawson et al., 2012). Consistently, the in vitro data presently obtained with our cholestasis assay resulted in similar SM values in rat and human SCH (Table 3). Thus, the in vitro cholestasis assay could overcome the inability of the present animal models to the detect cholestatic nature of drugs; in addition it can also indicate the species-specific sensitivity towards cholestatic action of the compounds. This model supports the use of sandwich-cultured human (but not rat) hepatocytes as preclinical model, as the correlation between SM and incidence is only observed in case of SCHH, but not in SCRH.

In conclusion, we have established a new in vitro model based on sandwich-cultured hepatocytes, to assess the potential of a compound to cause cholestasis by disturbing BA homeostasis. The model employs toxicity (reduced urea formation capacity) as an endpoint and has been validated with positive and negative control compounds in human hepatocytes; 8 out of 9 known cholestatic compounds (with previous reports of disturbing BA homeostasis (Fig. 6)) were flagged. Ritonavir, bosentan and treloandomycin were identified as compounds with potential cholestasis risk requiring further investigation. There were no compounds that were incorrectly flagged for cholestasis. We have shown with compounds like chlorpromazine, troglitazone, ticlopidine, that if a compound requires metabolism along with BA disposition disturbances to exert its toxicity, the model is competent to identify it as a potentially cholestatic compound. This model can give new insights into the toxicity mechanisms associated with different...
hepatotoxicants disturbing BA homeostasis. It carries the promise to decrease the use of laboratory animals for preclinical testing of drug-induced cholestasis.

Conflict of interest

There are no conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.taap.2013.10.032.

References


Troglitazone-induced intrahepatic cholestasis by an interference with the bile salt export pump (Bsep) by troglitazone and troglitazone sulfate. Toxicology 167, 89–98.


