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Prediction of the clinical risk of drug-induced cholestatic liver injury using an *in vitro* sandwich cultured hepatocyte assay

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Abbreviations: ALP, alkaline phosphatase; ALT, alanine transaminase; AST, aspartate transaminase; BA, bile acid; BSEP, bile salt export pump; C_{max}, maximum plasma concentration; $C_{max,u}$, maximum unbound plasma concentration; C_{ss}, steady state plasma concentration; DILI, drug-induced liver injury; γGT, gamma glutamyltranspeptidase; IC_{50}, half-maximal inhibitory concentration; LDH, lactate dehydrogenase; MRP, multidrug resistance-associated protein; PMDA, Pharmaceuticals and Medical Devices Agency; PK, pharmacokinetic; ROC, receiver operating characteristic; SCH, sandwich cultured hepatocyte; SEM, standard error of mean; TC, taurocholate; WME, Williams' Medium E

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Abstract

Drug-induced liver injury (DILI) is of concern to the pharmaceutical industry and reliable preclinical screens are required. Previously, we established an *in vitro* bile acid-dependent hepatotoxicity assay that mimics cholestatic DILI *in vivo*. Here, we confirmed that this assay can predict cholestatic DILI in clinical situations by comparing *in vitro* cytotoxicity data with *in vivo* risk. For 38 drugs, the frequencies of abnormal increases in serum alkaline phosphatase (ALP), transaminases, gamma glutamyltranspeptidase (γGT), and bilirubin were collected from interview forms. Drugs with frequencies of serum marker increases higher than 1% were classified as high DILI risk compounds. *In vitro* cytotoxicity was assessed by monitoring lactate dehydrogenase release from rat and human sandwich cultured hepatocytes (SCRHs and SCHHs) incubated with the test drugs (50 μM) for 24 h in the absence or presence of a bile acids mixture. Receiver operating characteristic analyses gave an optimal cutoff toxicity value of 19.5% and 9.2% for ALP and transaminases in SCRHs, respectively. Using this cutoff, high and low risk drugs were separated with 65.4–78.6% sensitivity and 66.7-79.2% specificity. Good separation was also achieved using

SCHHs. In conclusion, cholestatic DILI risk can be successfully predicted using a SCH-based assay.

Introduction

Drug-induced liver injury (DILI) is a potentially serious adverse reaction that leads to the dropout of candidate compounds from drug development processes and the withdrawal of pharmaceuticals from clinical use (Kaplowitz, 2013). DILI can severely damage the liver, resulting in liver transplantation in worst case scenarios; hence, it is essential to identify, remove, and/or assign alerts for possible DILI risk compounds at all stages of the drug development process.

The accumulation of bile acids (BAs) within hepatocytes has been suggested as an underlying mechanism of cholestatic DILI (Stieger et al., 2000; Fattinger et al., 2001; Byrne et al., 2002; Kostrubsky et al., 2006). The bile salt export pump (human BSEP/rat Bsep), which is localized on the apical side of the hepatocyte plasma membrane, plays a major role in the excretion of BAs from the liver to the bile (Meier and Stieger, 2002). Several genetic mutations of BSEP are associated with progressive familial intrahepatic cholestasis type 2, and cause severe intracellular accumulation of BAs within the liver (Strautnieks et al., 1998). A similar phenotype also occurs if BSEP function is inhibited or its expression is suppressed by drugs, leading to cholestatic or mixed type DILI (Roman et al., 2003; Dawson et al., 2012).

This concept is widely accepted; however, it is still difficult to detect cholestatic DILI risk using preclinical animals. Notably, *Bsep* knockout mice develop severe cholestasis when fed a BA-enriched diet, but display only mild cholestasis when fed a normal diet. This outcome is explained by the fact that (i) endogenous BAs are less toxic to rodents than humans because taurine-conjugated BA species (non-toxic) were predominant in rodents, while glycine-conjugated BAs (toxic) were predominant in human (Thomas et al., 2008; Marion et al., 2012), and (ii) adaptive changes in the expression levels of enzymes and transporters occur in *Bsep* knockout mice (Wang et al., 2003; Wang et al., 2009).

A membrane vesicle assay is considered as a simple and suitable alternative method of predicting the cholestatic DILI potential of test drugs. Previous studies have demonstrated that many drugs that cause cholestatic DILI are potent inhibitors of BSEP (Morgan et al., 2010; Dawson et al., 2012; Warner et al., 2012; Pedersen et al., 2013). Nonetheless, it was gradually indicated that the BSEP-based vesicle assay might misestimate the risk of cholestatic DILI, likely because cell-free systems lack drug metabolism pathways and other BA efflux transporters (such as multidrug resistance-associated protein 3 (MRP3) and MRP4) (Dawson et al., 2012).

Historically, predictions of cholestatic DILI risk have depended mainly on membrane vesicle assays as described above (Morgan et al., 2010; Dawson et al., 2012; Morgan et al., 2013; Pedersen et al., 2013; Kock et al., 2014). Although this method is easy to perform and suitable for high throughput screening, it does have some limitations, as discussed elsewhere (Morgan et al., 2013; Pedersen et al., 2013; Kock et al., 2014). It was also claimed that sandwich cultured hepatocytes (SCH)-based transport assays are more suitable than membrane vesicle assays to precisely differentiate BSEP inhibitors that result in relatively mild DILI from those associated with more severe DILI (Pedersen et al., 2013).

To overcome the less toxic nature of BA species in preclinical animals and the shortcomings of the membrane vesicle assay described above, we established a unique cell-based toxicity assay using SCHs in combination with titrated human BA species (Ogimura et al., 2011). The rationale of using SCHs is that drug metabolizing enzymes and transporters are well maintained compared to standard culture conditions (Swift et al., 2010). If a test drug and/or its metabolite inhibit BA efflux from SCHs, the accumulation of BAs eventually induces cell death and is detected by the release of lactate dehydrogenase (LDH) into the medium. Indeed, in a previous study,

we have demonstrated that 11 of the 26 test drugs examined exhibited significant toxicity in SCRHs only in the presence of such human BA compositions (Ogimura et al., 2011). Although most (8 of 11) of these toxic drugs were known BSEP/Bsep inhibitors, it is not yet known if our *in vitro* assay is capable of predicting cholestatic DILI risk in clinical situations; therefore, the aim of this study was to answer this question.

First, we performed an exhaustive search of the Japanese Adverse Drug Event Report database to refine test drug candidates, and then surveyed documents of interest to obtain the actual frequencies of serum marker increases. Finally, these data were compared with the *in vitro* toxicity data obtained using SCRHs and SCHHs.

Materials and Methods

Selection of test drugs

To select test drugs, we surveyed the Japanese Adverse Drug Event Report database, which is operated by Japan's Pharmaceuticals and Medical Devices Agency (PMDA). From a total of 1,866,993 cases (reported between August 2004 and August 2013), 421,904 were extracted as drug-related adverse events. From these cases, 1,984 drugs were identified as the most likely candidates, according to the guideline issued by the PMDA. The drugs of interest were scored according to the number of cases using the following keywords: hepatocellular injury (“liver injury”, “liver dysfunction”, “serum albumin concentration”, “total protein in serum”, “aspartate transaminase (AST)”, “alanine transaminase (ALT)”, “transaminase”, “non-alcoholic steatohepatitis”, “hepatic cirrhosis”, “liver inflammation”, “steatosis”, “acute or chronic liver failure”, “hepatic fibrosis”, “liver carcinoma”, and “fulminant hepatic failure”); and cholestasis (“jaundice”, “hyper bilirubinemia”, “alkaline phosphatase (ALP)” and “gamma glutamyltranspeptidase (γGT)”. The 243 drugs were then arranged in descending order of the rate of cholestatic DILI, which was calculated as follows: Rate of cholestatic DILI = (cases with cholestasis keywords) / ((cases with

cholestasis keywords) + (cases with hepatocellular injury keywords)). Finally, 38 drugs that covered a broad range of cholestatic DILI incidences were selected from the list. As for the test set in SCHHs, 6 drugs which showed strong hepatocyte toxicity independent of BAs in SCRHs were firstly excluded, and then represented 22 drugs (12 drugs which showed BA-dependent hepatocyte toxicity and 10 drugs which did not show any toxicity; in SCRHs) were selected from 38 drugs.

Frequency of serum test abnormalities

To calculate the frequencies of serum marker increases, we collected interview forms for 38 brand-name drugs from pharmaceutical companies. Abnormal serum levels of ALP, transaminases (AST/ALT), γGT, and bilirubin were selected as markers of a particular type of DILI. The incidences of these serum biomarkers and the numbers of patients (including infants) examined were extracted from the interview forms. The clinical studies based on (i) combination therapy (e.g., antibiotics with proton pump inhibitors) and (ii) non-Japanese patients, were excluded from the number of cases. The frequencies of increases in the markers were calculated as follows: Frequency of ALP, transaminases, γGT or bilirubin abnormalities (%) = (number of cases with the

serum test abnormality) / (number of patients enrolled in the clinical studies in Japan) × 100.

Materials

BAs and test compounds were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan), Sigma-Aldrich (St. Louis, MO, USA), or Calbiochem (Darmstadt, Germany). Williams' Medium E (WME), antibiotic-antimycotic solution, and GlutaMAX™ were purchased from Invitrogen (Carlsbad, CA, USA). Insulin was purchased from Sigma-Aldrich. Matrigel and ITS premix culture supplement were purchased from BD Biosciences (San Jose, CA, USA). Collagenase and dexamethasone were purchased from Wako Pure Chemical Industries Ltd. All other chemicals and solvents were of analytical grade, unless otherwise noted.

Animals

Male Sprague Dawley rats (SLC Japan Inc., Tokyo, Japan) aged 7–8-weeks were used throughout the study. The animals were treated humanely in accordance with the guidelines issued by the National Institutes of Health (Bethesda, MD, USA). In

addition, all procedures were approved by the Animal Care Committee of Chiba University (Chiba, Japan).

Rat and human sandwich cultured hepatocytes

Tissue culture (96-well) plates were pre-coated with type 1 collagen (BD Biosciences) at least 1 h prior to the preparation of hepatocyte cultures. Rat hepatocytes were isolated using a two-step perfusion method, as described previously by our group (Ogimura et al., 2011; Susukida et al., 2015). Isolated hepatocytes were seeded onto collagen (1.5 mg/mL, pH 7.4)-coated 96-well plates at a density of 0.48×10^5 cells/well in plating medium consisting of WME containing 5% FBS, 0.1 μM dexamethasone, 4 mg/L insulin, 2 mM GlutaMAX™, 15 mM HEPES, pH 7.4, penicillin (100 units/mL), and streptomycin (100 μg/mL). At 1.5 h after seeding, the medium was aspirated, and fresh plating WME was added to each well. Briefly, 24 h after plating, hepatocytes were overlaid with Matrigel (0.25 mg/mL) dissolved in ice-cold culture medium consisting of WME containing 1% ITS, 0.1 μM dexamethasone, 2 mM GlutaMAX™, penicillin (100 units/mL), and streptomycin (100 μg/mL). Thereafter, the medium (WME) was changed daily till 4 days after cell seeding. For SCHHs,

cryopreserved human hepatocytes (Hu1437, Hu1524 and Hu4197; Life Technologies, Grand Island, NY, USA) were thawed and seeded according to the manufacturer's protocol. Thawed hepatocytes were poured into CHRM® Medium (Life Technologies) at 37°C. The cells were centrifuged at 100 g for 10 min at room temperature and resuspended in plating medium consisting of WME containing 5% FBS, 0.1 µM dexamethasone, 4 mg/L insulin, 2 mM GlutaMAX™, 15 mM HEPES, pH 7.4, penicillin (100 units/mL), and streptomycin (100 µg/mL). Then hepatocytes were seeded onto collagen-coated 96-well plate at a density 0.48×10^5 cells/well. At 4 h after seeding, the medium was aspirated and hepatocytes were overlaid with Matrigel (0.25 mg/ml) dissolved in ice-cold culture medium. The medium (WME) was changed daily till 5 days after cell seeding. Both SCRHs and SCHHs were maintained at 37°C in a humidified atmosphere of 95% air/5% CO_2. Information of used lots of human hepatocytes is described in Table I

Cytotoxicity assay

SCRHs and SCHHs were exposed to each test compound in the presence or absence of a BA mixture comprising the 12 BAs shown in Table II. After exposure to

the test compounds for 24 h, cytotoxicity was assessed by measuring the activity of LDH released from damaged cells (LDH sample) using the LDH-Cytotoxic Test (Takara Bio Inc., Shiga, Japan). The LDH activity was expressed as a percentage of maximum LDH activity in Triton X-100-solubilized lysates of control SCHs (LDH Triton X-100), using the following equation: Cell toxicity (%) = (LDH sample - LDH blank) / (LDH Triton X-100 - LDH blank) × 100. The LDH blank value was determined using untreated SCHs.

Receiver operating characteristic analysis

Statistical analyses of the predictabilities of the drug-induced frequencies of ALP, transaminases, γGT, and bilirubin increases in human serum were performed using receiver operating characteristic (Another) curves in GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA). The ROC curves were generated by computing the paired true-positive and false-positive rates for all possible thresholds of the cell toxicity assays using SCRHs and SCHHs. In ROC curves, the predictions that occur towards the top-left corner are indicative of high true-positive and low

false-positive rates. This approach generates a threshold where the false-positive rate is zero and the true-positive rate is a fraction between 0 and 100%.

Results

Investigation of the clinical risk of cholestatic DILI compounds

To identify the test drugs, we surveyed the PMDA's voluntary adverse reaction database. From the top 243 drugs related to DILI, 38 were arbitrary selected to cover the whole range and a detailed survey of the clinical frequencies of cholestatic DILI was performed using the information in interview forms (Table III). The frequency of ALP increases ranged from 0.011% (clarithromycin) to 14.3% (clozapine), with a median of 0.52%. The frequency of transaminases (ALT/AST) increases ranged from 0.019% (clarithromycin) to 50.0% (bosentan), with a median of 1.85%. The frequency of γGT increases ranged from 0.0040% (tranilast) to 18.1% (carbamazepine), with a median of 0.61%. The frequency of bilirubin increases ranged from 0.0040% (clarithromycin) to 2.5% (bosentan), with a median of 0.19%. Notably, the frequencies of ALP and transaminases (these serum markers are used in the diagnosis and management of DILI (Chalasani et al., 2014)) were positively correlated (linear regression after logarithmic conversions; r^2 = 0.840 for ALP versus transaminases (Fig. 1)). The other relationships between frequencies of clinical markers (e.g., ALP versus γGT) were shown in Supplementary Fig.1.

Optimization of the BA mixture concentration

Before performing BA-dependent drug toxicity assays, we examined the sensitivities of SCRHs and SCHHs to various concentrations of the standard (1×) BA mixture of human serum (Scherer et al., 2009) (Table II). In both cell types, toxicity began to rise at 100× concentrations, and increased in a concentration dependent manner. In our assay condition, SCRH was more susceptible compared to SCHH (Fig. 2). To detect subtle changes in BA accumulation-dependent enhancement of the toxicity, we used an 115× BA mixture for SCRH and an 150x BA mixture for SCHH in subsequent assays, where the basal toxicity of 20-30% were expected. Although the optimized exogenous BAs to SCRHs and SCHHs in this study were significant excess (at least 115x human serum concentrations), we have already reported the mRNA expression levels of BA uptake transporters (i.e., Ntcp and Oatp1a1) are dramatically diminished in SCRHs and the downregulation of uptake transporters supports our rationale for employing an extremely high concentration range for total BAs (227–681 μM) compared with BA levels observed in clinical situations (Susukida et al., 2015).

Evaluation of drug toxicity in SCRHs and SCHHs

The toxicities of the 38 drugs to SCRHs were determined using drug concentrations of 50 μM (except cyclosporine A used as positive control; 10 μM) and the 115× BA mixture. With the exceptions of 7 drugs (amiodarone, clozapine, duloxetine, ethinylestradiol, everolimus, fluoxetine, and sertraline), the drugs showed minimum toxicity to SCRHs in the absence of the BA mixture (Fig. 3A). The toxicities of the drugs were more or less enhanced in the presence of the 115× BA mixture (Fig. 3A). Similarly, the toxicities of the arbitrary selected 22 drugs to SCHHs were determined using drug concentrations of 50 μM (except cyclosporine A used as positive control; 10 μM) and the 150× BA mixture. With the exceptions of 2 drugs (amiodarone and tacrolimus, in Hu4197), the drugs showed minimum toxicity to SCHHs in the absence of the BA mixture (Fig. 3B-D). The toxicities of the drugs to SCHHs were more or less enhanced in the presence of the 150x BA mixture (Fig. 3B-D). When correlation analyses of the toxicities to each lot of human hepatocytes in the absence and presence of the BA mixture of tested 22 drugs were performed, a positive correlation was observed in the presence of BAs (Supplementary Figs. 2D-F) but not in the

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absence of BAs (Supplementary Figs. 2A-C). However, some drugs (clopidogrel, leflunomide and ticlopidine) showed lot variation for BA-dependent hepatocyte toxicity in SCHHs.

Correlation of rat and human sandwich hepatocyte toxicity data

To determine if the SCRHs and SCHHs responded similarly to the drugs, correlation analyses of the toxicities to each cell type in the absence and presence of the BA mixture were performed for the 22 drugs tested in common (Fig. 4). Although the correlation between the rat and human cells was poor in the absence of the BA mixture (Fig. 4A-C), a positive correlation was observed in the presence of this mixture (Fig. 4D: $y = 0.4492x - 2.142$, $r^2 = 0.7516$; 4E: $y = 0.6525x + 4.501$, $r^2 = 0.7543$; 4F: $y = 0.6242x + 3.698$, $R^2 = 0.7431$).

Prediction of clinical risk from the *in vitro* data

To evaluate if the *in vitro* toxicity data reflected the clinical risk of cholestatic DILI, the relationships between the *in vitro* and *in vivo* data were examined. Most of the drugs with lower frequencies of clinical ALP and transaminases increases showed minimal

BA-dependent drug toxicity to SCRHs, while those with higher frequencies of clinical ALP and transaminases increases showed enhanced BA-dependent toxicity (Figs. 5A and E). Similar tendencies were also observed when the frequencies of the ALP and transaminases increases were plotted against the BA-dependent toxicities of the drugs to SCHHs (Figs. 5B-D and 5F-H). To evaluate the usefulness of the *in vitro* assay further, ROC analysis was performed. The clinical increases in serum markers of 1.0% were used to separate the drugs into low and high risk groups. As a result, by setting an *in vitro* toxicity cutoff value of 19.5% for SCRHs, the drugs with a high risk of clinical ALP increases (>1.0%) were correctly sorted with 78.6% sensitivity and 79.2% specificity (Fig. 5A and Table IV). Similarly, by setting an *in vitro* toxicity cutoff value of 9.2% for SCRHs, the drugs with a high risk of clinical transaminases increases (>1.0%) were correctly sorted with 65.4% sensitivity and 66.7% specificity (Fig. 5E and Table IV). The study of SCRHs was replicated at least two times, and therefore the represented data was shown. In the replicated trial in SCRHs, it successfully predicted the clinical frequencies of serum marker increases for most drugs and therefore the reproducibility of the work has been established (data not shown). In SCHHs, approximately 70% sensitivity and 80% specificity for the

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prediction of both clinical ALP increases and transaminases (>1.0%) increases were obtained from the analyses of Hu1524 and Hu4197 toxicity data. (Figs. 5C-D, 5G-H and Table IV). However, analysis of the other lot of SCHHs (Hu1437) toxicity data resulted in poor sensitivity (less than 50% for both serum markers), because of the lot variation of clopidogrel, leflunomide and ticlopidine-induced BA-dependent hepatocyte toxicity (Figs. 5B, F and Table IV). The clinical frequency of γGT increase was also well predicted in both SCRHs and SCHHs (Supplementary Figs. 3A-D, and Supplementary Table II). On the contrary, the frequency of serum bilirubin increase was not correlated with the *in vitro* toxicities in both SCRHs and SCHHs (Supplementary Figs. 3E-H, Supplementary Table II). Prediction of serum marker elevations was not possible in the absence of the BA mixture (Supplementary Fig. 4).

To determine if the predictability of the *in vitro* assay was improved by considering the drug concentration *in vivo*, the maximum plasma concentrations (C_{max}) and maximum unbound plasma concentrations ($C_{max,u}$) were collected from the interview forms (Supplementary Table I). These concentrations were multiplied by the *in vitro* toxicity data obtained in SCRHs and SCHHs and plotted against the *in vivo* frequencies of the increases in serum markers. However, such corrections did not

improve the predictive capability of the *in vitro* assay (compare Supplementary Figs. 5A and B with Fig. 5).

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Discussion

We have previously established a unique SCH-based toxicity assay system focusing on cholestatic DILI (Ogimura et al., 2011) and now successfully confirmed here that the assay is particularly useful for the prediction of clinical serum marker abnormalities. The SCH-based toxicity assay described here has an advantage of including metabolic enzymes and BA efflux transporters other than BSEP/Bsep, over the membrane vesicle transport assay. For some drugs, their metabolites inhibit BSEP/Bsep more potently than the parent compound. For example, the IC_{50} of troglitazone against [^{3}H]TC uptake into isolated bile canalicular membrane vesicles from rat liver is 3.9 μM, while that of the sulfated conjugate is as low as 0.4–0.6 μM (Funk et al., 2001). Exposure of SCRHs to 10 μM troglitazone enhances BA-dependent toxicity significantly (Ogimura et al., 2011), which is likely explained by the extensive metabolism of the drug to its sulfate conjugate in these cells (Izaki et al., submitted) (Yang and Brouwer, 2014). Considering the importance if metabolic enzymes in our SCH-based toxicity assay, it sometimes gives distinct result because of the presence of large interspecies differences in drug-metabolizing enzyme expression and activity. Our group has reported that the parent form of glibenclamide

(GLM, not used in the current study) might contribute to the BA-dependent hepatocyte toxicity in SCRHs (Ogimura et al., 2011) but it did not show BA-dependent hepatocyte toxicity in SCHHs (data no shown). That might be explained by the fact that GLM was more extensively metabolized in human liver microsomes, than in rat liver microsomes (Ravindran et al., 2013).

The BA-dependent hepatocyte toxicity of some drugs (clopidogrel, leflunomide and ticlopidine) in Hu1437 was lower than that of other two lots (Fig. 3B, Supplementary Fig. 2D-E) and that resulted in poor sensitivity of the frequencies of *in vivo* serum test abnormalities (Figs. 5B, F; Supplementary Figs. 3B, F; Table IV and Supplementary Table II). Given the parent form of these drugs showed minimum inhibitory effect against human BSEP ((Morgan et al., 2013), see Supplementary Table I), those metabolites possibly contribute to the toxicity and their intracellular amount might depend on the activity of metabolic enzymes in each lot. Notably, these three drugs are known to be metabolized by cytochrome P450 (CYP) 2C19 and their metabolites themselves have pharmacology effects (Bohanec Grabar et al., 2009; Farid et al., 2010; Nakkam et al., 2015), implying that CYP2C19 may be the candidate for producing toxic metabolites. According to the donor information of

human hepatocytes (Table I), the donor of Hu1437 was elderly (70 years old) Caucasian female. There was a clinical investigation in Caucasian for the influences of age and gender on the disposition of the CYP2C19 substrate and it reported that elderly women (i.e., the donor of Hu1437) may have lower activity of CYP2C19 (Hooper and Qing, 1990; Kobayashi et al., 2004). Therefore, that could be one of considerable reasons for the lower BA-dependent hepatocyte toxicity of these drugs (clopidogrel, leflunomide and ticlopidine) not appeared in this lot. Interestingly, same tendency was also observed in SCRHs. These drugs did not show BA-dependent hepatocyte toxicity in another trial of SCRHs (Supplementary Figs. 8A). Although it is unknown which CYP enzymes are involved in producing those toxic metabolites in rats, their amount may be influenced by the expression level of the involved CYP enzyme in each trial of SCRHs.

The results of other drugs except clopidogrel, leflunomide, and ticlopidine presented here did not show both interspecies and lot variation; indicating that rat cells were a good alternate for human cells. This finding is partially attributable to the similar inhibition profiles of human BSEP and rat Bsep, as reported previously (Morgan et al., 2010; Dawson et al., 2012). In a study of 56 compounds, it was found

that the IC_{50} values of the majority of compounds towards [^{3}H]TC uptake into membrane vesicles from Sf9 cells expressing human BSEP and rat Bsep were quite similar (Morgan et al., 2010). Moreover, in a study of 85 compounds, it was confirmed a close correlation (r^2 = 0.94) between the IC_{50} values of drugs towards [^{3}H]TC uptake into membrane vesicles isolated from Sf21 cells expressing human BSEP and rat Bsep (Dawson et al., 2012).

One might expect that the prediction accuracy of an *in vitro* assay would be improved by considering the pharmacokinetic (PK) parameters of each drug. In a previous study, 95% of compounds with a C_{ss}/BSEP IC_{50} ratio ≥ 0.1 were correctly identified as having an association with DILI, while the prediction accuracy was as low as 79% when considering the BSEP IC_{50} values alone (Morgan et al., 2013). However, consideration of the C_{max} or $C_{max,u}$ values did not improve the predictability of the *in vitro* assay described here (Supplementary Figs. 5A and B). A possible reason for this finding is the variable accumulation of each drug inside hepatocytes *in vivo* (Grime et al., 2008). Alternatively, patients exhibiting DILI might have quite different exposures to non-DILI patients. Given this situation, PK parameters determined using non-DILI patients might not improve the predictabilities of assays.

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Similarly, it was suggested that correction of assay results using PK parameters did not improve the prediction accuracy (Morgan et al., 2013).

We do not have a clear basis for setting the test drug concentration to 50 μM, but this value is empirically chosen based on previous studies (Morgan et al., 2010; Morgan et al., 2013; Pedersen et al., 2013; Aleo et al., 2014; Kock et al., 2014). For example, the inhibitory effects of 250 compounds on human BSEP in a membrane vesicle transport assay and found that 86 compounds inhibited human BSEP function significantly ($P < 0.05$) at 50 μM (Pedersen et al., 2013). To verify the detectability of 50 μM of the test drug concentration in our SCH-based toxicity assay, the concentration-response relationships in SCRHs were tentatively examined for two selected groups; (i) 8 drugs (atorvastatin, clopidogrel, cyclosporine A, flutamide, leflunomide, naftopidil, tacrolimus, and ticlopidine) which showed BA-dependent hepatocyte toxicity at 50 μM and (ii) 9 drugs (carbamazepine, clarithromycin, lamivudine, levofloxacin, ranitidine, tranilast, valproate, valsartan, and voriconazole) which did not show BA-dependent hepatocyte toxicity at 50 μM but their 100 x $C_{max,u}$ are beyond 50 μM. As a result, calculated LC_{50} (half-maximal lethal concentration) values of drugs in group (i) were set within the range of 6.0-128 μM while those in

group (ii) were within the range of 183-4380 μM (Supplementary Figs. 8A and B). It was found that drugs with LC_{50} < 400 μM apparently showed detectable BA-dependent hepatocyte toxicity in SCRHs under setting the test drug concentration to 50 μM; however, the toxicity of drugs with LC_{50} < 60 μM seems saturated while the toxicity of drugs with LC_{50} > 500 μM seems overlooked, implying that drugs with extremely lower or higher LC_{50} values could not be properly evaluated by fixing the test drug concentration (Supplementary Fig. 8C). From the theoretical stand point of view, LC_{50} values would be more comprehensive index than the cell toxicity data obtained at fixed drug concentration. It is to be determined in the future study if predictability is improved by the use of LC_{50} values in combination with PK parameters.

Our group also found that efflux transporters other than Bsep might be involved in exporting some BAs from hepatocytes, and that their inhibition aggravates BA-dependent toxicity in SCRHs (Susukida et al., 2015). Flutamide is an example of one such drug that inhibits other efflux transporters. The IC_{50} of flutamide towards human BSEP and rat Bsep was reported to be as high as 143.2 μM and 78.7 μM, respectively (Dawson et al., 2012), and 50 μM flutamide did not inhibit human BSEP

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in a previous study (Pedersen et al., 2013). However, here, flutamide induced BA-dependent toxicity in both human and rat SCHs (Fig. 3), implying the involvement of the inhibition of BA efflux proteins other than BSEP/Bsep. In line with this proposal, it has been already suggested that inhibition assays focusing on BSEP only are insufficient, and highlighted the need to consider basolateral efflux transporters such as MRP3 and MRP4 or another unidentified BA transporter for accurate predictions of drug toxicities (Morgan et al., 2013; Kock et al., 2014; Susukida et al., 2015). In fact, the human BSEP inhibition data alone (IC_{50} values in BSEP-expressed membrane vesicles, see Supplementary Table I) did not correlate well with abnormal frequencies of serum markers (data not shown).

Other studies categorized DILI risk based on FDA risk classifications, including the Black Box Warning, Warning / Precaution, Adverse Reaction, and Not Mentioned labels. These classifications are not determined solely by the frequency of the marker increase, but also take other factors into account (Avigan, 2014), such as the estimated rates of life-threatening hepatotoxic adverse events among treated patients, the presence (or absence) of effective tools to reduce DILI risk in treated patients, and regulatory outcomes. Notably, the SCH assay described here simply

reflects the probability of BA accumulation inside hepatocytes and subsequent cell death. From this stand point, we think it is reasonable that the assay does not necessarily predict the FDA category classification, but can predict the frequencies of serum test abnormalities (Supplementary Fig. 6). However, some false-negative or false-positive predictions were obtained (Fig. 5). One of the possible reasons is the *in vivo*-specific effects, such as the induction of marker enzymes (ALP and γGT), is responsible for the false-negative cases (e.g., carbamazepine) (Voudris et al., 2005). Moreover, correlation between the frequency of serum bilirubin increase and the frequencies of other serum marker increases in patients are relatively poor (r^2 = 0.2581-0.3144) (Supplementary Figs. 1C-E), and the frequency of serum bilirubin increase was not well correlated with cytotoxicity in both SCRHs and SCHHs, compared with other serum markers (Supplementary Figs. 3E-H, Supplementary Table II). One of the considerable reasons why the clinical frequency of serum bilirubin increase did not give good predictability for the risk of cholestatic DILI might be that bilirubin was excreted to the bile by MRP2 but not BSEP (Jedlitschky et al., 1997). Our SCH-based toxicity assay can evaluate the hepatocyte toxicity due to intracellular BA accumulation by mainly BSEP inhibition, and therefore the serum

marker elevated by different mechanisms (i.e., MRP2 inhibition) may not be predicted with our assay system.

In conclusion, BA-dependent cytotoxicity assay using SCHs might be a useful preclinical screening tool to predict the risk of cholestatic DILI. Recently, it was reported that the prediction accuracy of general DILI risk is improved when considering the potential of mitochondrial dysfunction as well as BSEP inhibition (Aleo et al., 2014). These observations are consistent with the understanding that BA-induced apoptosis is one of the major causes of hepatocellular injury (Woolbright and Jaeschke, 2012). From a practical point of view, the assay described here seems beneficial because it does not require knowledge of the clinical dose or target concentration, which are sometimes difficult to estimate during the preclinical drug development stage.

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Authorship Contributions

Participated in research design: Susukida, Sekine, Nozaki, and Ito

Conducted experiments: Susukida, Nozaki and Tokizono

Contributed new reagents or analytic tools: Susukida, Sekine, Nozaki, and Ito

Performed data analysis: Susukida, Sekine, Nozaki, Tokizono, and Ito

Wrote or contributed to the writing of the manuscript: Susukida, Sekine, Nozaki, and Ito

Conflict of Interest

The authors state no conflicts of interests.

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Footnotes

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Figure Legends

Fig. 1. Relationships between the frequencies of serum marker increases in patients.

Frequencies of increases in the serum levels of ALP and transaminases. The frequencies were calculated for 38 selected drugs based on the numbers of cases reported in the interview forms. Frequency data are shown in Table III.

Fig. 2. BA concentration-dependent toxicity in SCRHs and SCHHs.

SCRHs (open circles) and SCHHs (open squares) were cultured for 4 days (rat) or 5 days (human) then treated with various concentrations of the BA mixture (0-150x standard BA mixture for SCRH and 0–250× standard BA mixture for SCHHs) for 24 h. The composition and concentration of the standard (1×) BA mixture is shown in Table II. Cell toxicity was determined by measuring the activity of LDH released into the medium. Data are represented as the mean ± standard error of mean (SEM) of n = 3.

Fig. 3. *In vitro* toxicities of the selected drugs to SCRHs and SCHHs in the absence or presence of the BA mixture.

The SCRHs were cultured for 4 days (A) and SCHHs were cultured for 5 days (B, Hu1437; C, Hu1524; D, Hu4197). They were treated with cyclosporine A (10 μM) or the other test drugs (50 μM) in the absence (open bars) or presence (closed bars) of the BA mixture (115x for SCRH and 150× for SCHH). The basal toxicities in the absence and presence of the BA mixture were obtained and subtracted from the corresponding data obtained in the presence of the test drugs. Data are represented as the mean ± SEM of n = 3 for SCRH and n=2-3 for SCHH.

Fig. 4. Correlation between the *in vitro* BA-dependent toxicities of the selected drugs to SCRHs and SCHHs.

The *in vitro* toxicities of the common 22 drugs to SCRHs and SCHHs in the absence (A-C) or presence (D-F) of the BA mixture. Inset of each graph (D-F) shows drugs sitting on the x- and y-axes in the enlarged scale (within 0-15% of cell toxicity; drug numbers correspond to those are shown in Table III). The original data are shown in Figure 3. A positive correlation (D: $y = 0.4492x – 2.142$, $r^2 = 0.7516$; E: $y = 0.6525x + 4.501$, $r^2 = 0.7543$; F: $y = 0.6242x + 3.698$, $r^2 = 0.7431$) was observed in the presence of the BA mixture.

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Fig. 5. Relationship between the frequencies of serum marker increases *in vivo* and BA-dependent toxicity *in vitro* in the presence of BA mixture.

(A–H) The frequencies of increases in the serum levels of ALP (A-D), and transaminases (E-H) versus the *in vitro* toxicities to SCRHs (A and E) and SCHHs (B-D and F–H). The vertical dotted lines represent the borderline frequencies of 1%. The horizontal dotted lines represent the cutoff values determined by the ROC analysis, which gave the best separation of high and low risk drugs (see Table IV for details). Drug numbers correspond to those shown in Table III.

Table I. Donor information of used lots of human hepatocytes

Lot number	Sex	Age	Race
Hu1437	F	70 years old	Caucasian
Hu1524	M	58 years old	Caucasian
Hu4197	F	31 years old	Caucasian

Table II. Composition of the 1× BA mixture standard. Concentrations of each BA are set based on the standard BA constituents of human serum (Scherer et al., 2009).

Bile acid	1× standard concentration (μM)
Cholic acid	0.30
Chenodeoxy cholic acid	0.50
Glycochenodeoxycholic acid	2.60
Deoxycholic acid	1.10
Lithocholic acid	0.045
Ursodeoxycholic acid	0.17
Glycocholic acid	0.62
Glycodeoxycholic acid	0.57
Taurocholic acid	0.070
Taurochenpodeoxycholic acid	0.32
Taurolithocholic acid	0.13
Tauroursodeoxycholic acid	0.43

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Table III. List of the drugs used in the study and their *in vivo* profiles and *in vitro* toxicities obtained in SCH assays.[1)] The frequencies of serum marker abnormalities were calculated based on the data included in the interview forms. (Number of cases with the serum test abnormality) / (number of patients enrolled in the clinical studies in Japan) was shown at the right of each percentage.[2)] LDH release in the absence ((-)BA) or presence ((+)BA) of the BA mixture. Basal toxicity obtained in the absence of the drug was subtracted. NT: not tested.

No	Drug	Frequency of serum marker increase (%)[1)]				SCRH toxicity (%)[2)]		SCHH (Hu1437) toxicity (%)[2)]		SCHH (Hu1524) toxicity (%)[2)]		SCHH (Hu4197) toxicity (%)[2)]	
		ALP	Transaminases	γGT	Bilirubin	(-) BA	(+) BA	(-) BA	(+) BA	(-) BA	(+) BA	(-) BA	(+) BA
1	Acarbose	0.24 (11/4543)	3.00 (136/4543)	0.44 (20/4543)	NT	1.5	9.8	NT	NT	NT	NT	NT	NT
2	Amiodarone	0.22 (3/1352)	1.63 (22/1352)	0.22 (3/1352)	0.074 (1/1352)	28.8	78.0	1.1	34.6	2.5	49.2	48.9	55.6
3	Atorvastatin	1.26 (72/5702)	5.28 (301/5702)	3.56 (203/5702)	0.37 (21/5702)	0.5	64.2	0.6	29.5	0.3	55.1	0.0	34.2
4	Bosentan	7.5 (3/40)	50 (20/40)	10.0 (4/40)	2.5 (1/40)	0.0	23.4	NT	NT	NT	NT	NT	NT
5	Carbamazepine	5.54 (18/325)	12.2 (41/335)	18.1 (53/293)	NT	0.0	0.0	0.0	0.0	0.0	0.0	0.0	7.2
6	Clarithromycin	0.011 (3/26923)	0.019 (5/26923)	0.015 (4/26923)	0.004 (1/23029)	0.0	15.5	NT	NT	NT	NT	NT	NT
7	Clopidogrel	2.87 (65/2268)	9.17 (208/2268)	4.59 (104/2268)	1.082 (38/3511)	0.0	70.2	0.0	0.0	0.0	19.1	0.0	10.9
8	Clozapine	14.3 (11/77)	49.4 (38/77)	15.6 (12/77)	NT	76.3	81.1	NT	NT	NT	NT	NT	NT
9	Cyclosporine A	1.53 (112/7300)	2.47 (180/7300)	0.53 (39/7300)	0.87 (61/6980)	9.8	78.6	0.0	41.2	0.0	72.4	0.0	48.7
10	Duloxetine	2.90 (36/1242)	11.0 (136/1242)	3.62 (45/1242)	2.01 (25/1242)	86.5	85.4	NT	NT	NT	NT	NT	NT
11	Ethinylestradiol	5.28 (36/682)	1.88 (14/743)	0.82 (6/730)	0.84 (8/955)	89.9	82.1	NT	NT	NT	NT	NT	NT
12	Everolimus	2.00 (25/1247)	11.6 (145/1247)	3.61 (45/1247)	0.24 (3/1247)	86.5	88.5	0.0	40.8	0.0	64.1	1.6	56.1
13	Famotidine	0.065 (13/20137)	0.39 (78/20137)	0.065 (13/20137)	0.055 (11/20137)	0.0	0.8	0.0	0.0	0.0	0.0	0.0	3.6
14	Fexofenadine	0.013 (1/7838)	0.24 (19/7838)	0.064 (5/7838)	0.137 (6/4367)	0.4	0.9	3.4	0.0	2.9	0.0	0.0	0.0
15	Fluoxetine	0.73 (4/550)	1.82 (10/550)	1.09 (6/550)	1.64 (9/550)	84.1	90.1	NT	NT	NT	NT	NT	NT
16	Flutamide	3.14 (201/6393)	26.5 (1692/6393)	5.91 (378/6393)	0.53 (34/6393)	1.2	84.9	0.0	49.7	0.0	65.5	0.0	86.3
17	Fluvoxamine	0.73 (7/965)	6.84 (66/965)	2.38 (23/965)	0.140 (1/712)	0.0	0.0	NT	NT	NT	NT	NT	NT
18	Lamivudine	1.23 (40/3253)	2.55 (83/3253)	3.60 (117/3253)	2.21 (72/3253)	0.1	0.0	0.0	0.0	0.0	0.0	0.0	10.6
19	Leflunomide	5.21 (19/365)	6.58 (24/365)	7.67 (28/365)	0.057 (1/1704)	0.9	34.4	0.0	0.0	0.0	43.4	0.0	51.1
20	Levofloxacin	0.041 (13/31810)	0.35 (111/31810)	0.075 (24/31810)	0.053 (17/31810)	0.0	0.6	0.0	0.0	1.9	7.8	0.0	0.0
21	Losartan	0.029 (11/36288)	0.21 (76/36288)	0.077 (29/36288)	1.38 (15/1088)	2.5	6.6	0.0	0.0	2.7	0.0	0.0	0.0
22	Methotrexate	0.55 (22/4038)	1.61 (65/4038)	0.15 (6/4038)	0.49 (21/4321)	1.1	4.2	0.0	0.0	3.8	17.9	1.7	0.0
23	Mitiglinide	0.84 (13/1555)	3.73 (58/1554)	3.23 (50/1550)	0.83 (13/1559)	0.2	6.6	NT	NT	NT	NT	NT	NT
24	Naftopidil	0.027 (6/22013)	0.27 (60/22013)	0.03 (6/22013)	NT	0.5	76.8	NT	NT	NT	NT	NT	NT
25	Pioglitazone	0.48 (23/4776)	2.34 (112/4776)	0.92 (44/4767)	0.19 (9/4789)	0.7	0.0	0.0	0.0	0.0	14.2	3.8	3.9
26	Pranlukast	0.032 (3/9240)	0.13 (12/9240)	0.022 (2/9240)	0.162 (15/9240)	0.0	5.2	NT	NT	NT	NT	NT	NT
27	Pravastatin	0.13 (15/11137)	1.08 (120/11137)	0.30 (33/11137)	0.081 (9/11137)	1.7	0.7	0.0	0.0	0.0	0.2	1.9	6.1
28	Ranitidine	0.063 (10/15761)	0.36 (96/15761)	0.082 (13/15761)	NT	0.0	8.5	NT	NT	NT	NT	NT	NT
29	Rosuvastatin	0.20 (18/8997)	1.45 (130/8997)	0.68 (61/8997)	0.12 (22/19175)	2.6	1.9	0.0	0.0	0.0	2.7	0.0	8.6
30	Sertraline	0.71 (9/1263)	7.52 (95/1263)	3.56 (45/1263)	1.35 (17/1263)	100.3	92.2	NT	NT	NT	NT	NT	NT
31	Simvastatin	0.22 (23/10420)	1.35 (141/10420)	0.45 (47/10420)	0.086 (9/10420)	0.2	11.0	0.0	11.1	0.0	45.4	3.2	22.2
32	Tacrolimus	1.07 (107/10038)	2.32 (233/10038)	1.60 (161/10038)	0.33 (33/10038)	1.4	79.9	5.1	42.3	0.6	56.6	18.3	53.2
33	Ticlopidine	0.15 (12/7933)	0.73 (58/7933)	0.16 (13/7933)	NT	0.0	39.8	0.0	0.0	0.0	15.5	0.0	33.4
34	Tranilast	0.13 (32/24788)	0.33 (81/24788)	0.004 (1/24788)	NT	0.0	6.2	0.0	0.0	0.0	0.0	0.0	0.0
35	Ursodeoxycholic acid	0.12 (12/9880)	0.28 (28/9880)	0.061 (6/9880)	0.04 (4/9880)	6.5	11.1	NT	NT	NT	NT	NT	NT
36	Valproate	0.35 (19/5366)	0.70 (53/5366)	0.093 (5/5366)	0.019 (1/5366)	0.0	0.0	NT	NT	NT	NT	NT	NT
37	Valsartan	0.24 (19/7814)	1.11 (88/7814)	0.47 (37/7814)	0.13 (10/7814)	0.0	11.9	NT	NT	NT	NT	NT	NT
38	Voriconazole	7.00 (7/100)	13.0 (13/100)	11.0 (11/100)	0.05 (1/1921)	0.3	8.2	0.0	0.0	0.0	0.4	0.0	1.4

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Table IV. Predictabilities of the frequencies of *in vivo* serum test abnormalities from *in vitro* toxicity assays. Drugs (38 drugs for SCRH and 22 drugs for SCHH) were divided into two groups with lower or higher than 1.0% frequency of serum markers. A ROC analysis was performed to generate the best separation of the two groups using the *in vitro* drug toxicity data obtained in the presence of BA mixture. For example, by setting an *in vitro* toxicity cutoff value of 19.5%, drugs with a higher risk of ALP increase were correctly predicted with 78.6% accuracy, while drugs with a lower risk of ALP increase were correctly predicted with 79.2% accuracy.

		Serum marker	
		ALP	Transaminases
SCRH	In vitro toxicity cutoff (%)	19.5	9.2
	Sensitivity (%)	78.6	65.4
	Specificity (%)	79.2	66.7
	Area under the ROC curve	0.738	0.681
	P value	0.0155	0.076
SCHH (Hu1437)	In vitro toxicity cutoff (%)	20.3	5.6
	Sensitivity (%)	50.0	43.8
	Specificity (%)	91.7	100.0
	Area under the ROC curve	0.700	0.719
	P value	0.1136	0.1217
SCHH (Hu1524)	In vitro toxicity cutoff (%)	18.5	11.0
	Sensitivity (%)	70.0	68.8
	Specificity (%)	83.3	83.3
	Area under the ROC curve	0.742	0.844
	P value	0.0560	0.0150
SCHH (Hu4197)	In vitro toxicity cutoff (%)	9.6	6.7
	Sensitivity (%)	80.0	80.0
	Specificity (%)	75.0	85.7
	Area under the ROC curve	0.825	0.857
	P value	0.0102	0.0082

Fig. 1

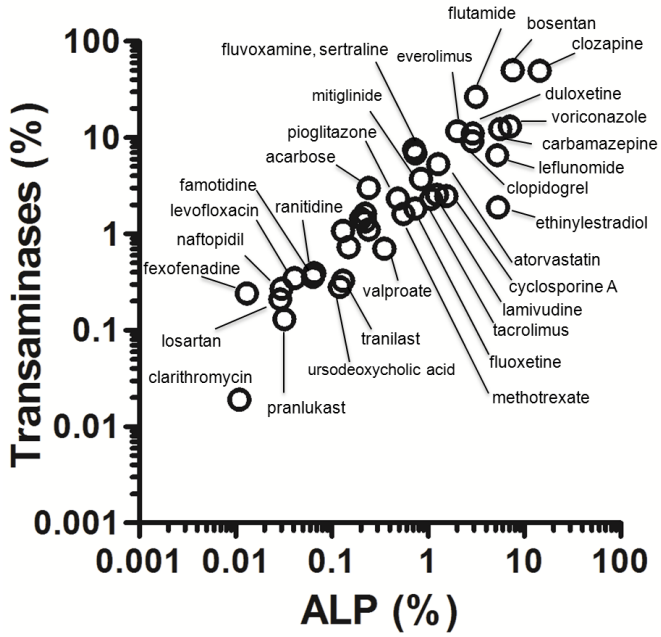


Fig. 2

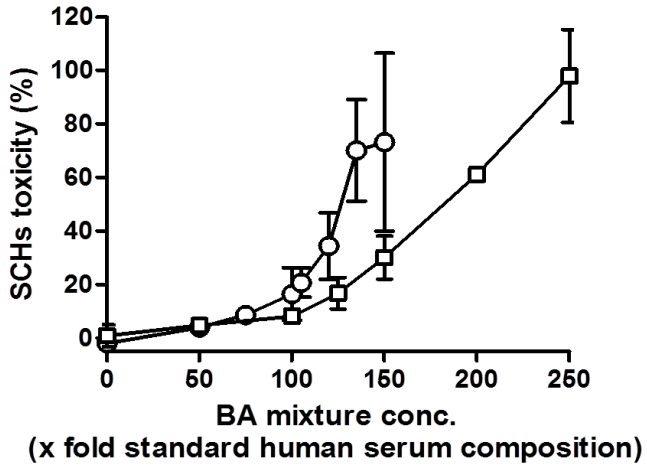


Fig. 3

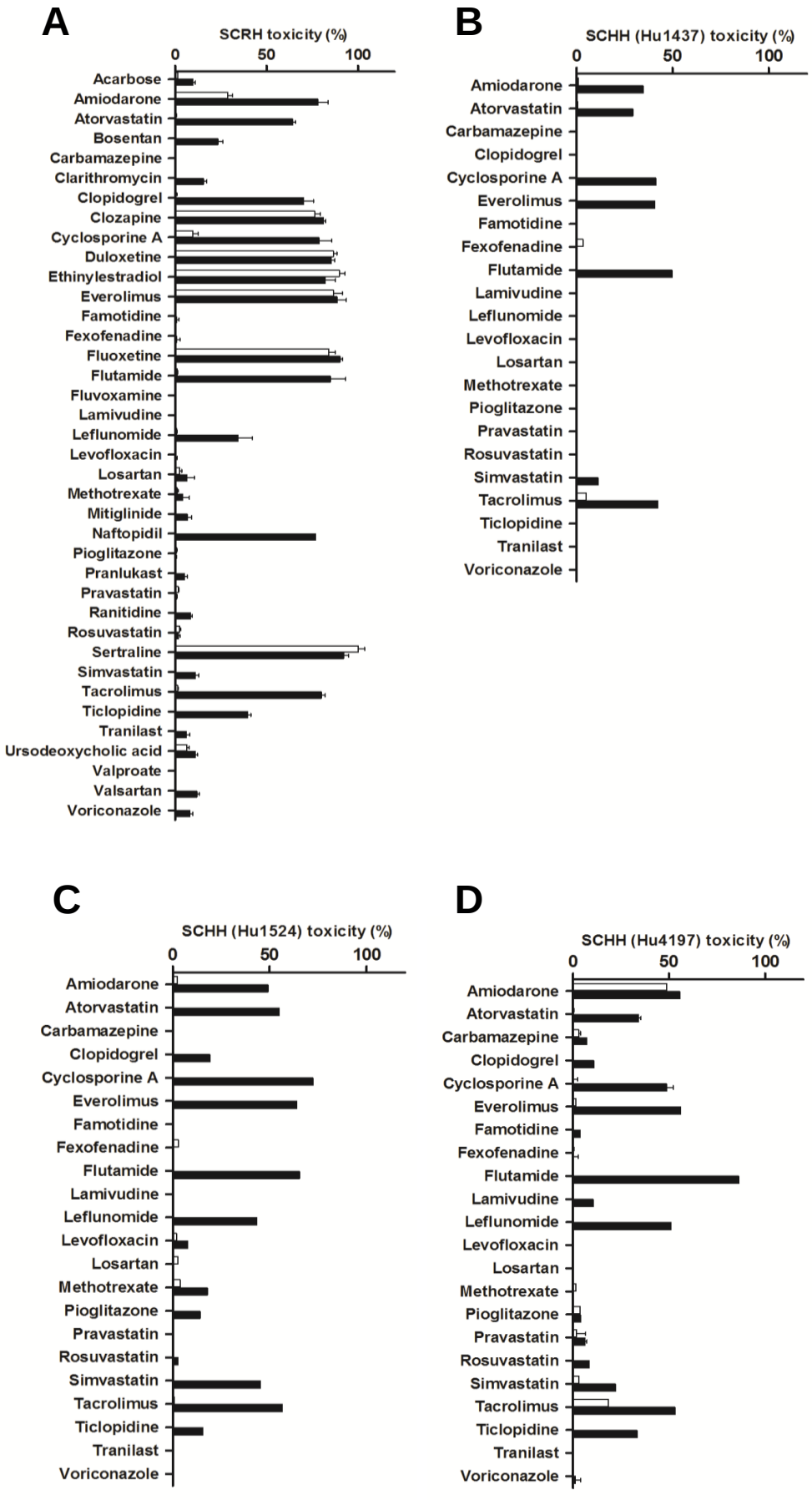

A
SCRH toxicity (%)
0
50
100
Acarbose
Amiodarone
Atorvastatin
Bosentan
Carbamazepine
Clarithromycin
Clopidogrel
Clozapine
Cyclosporine A
Duloxetine
Ethinylestradiol
Everolimus
Famotidine
Fexofenadine
Fluoxetine
Flutamide
Fluvoxamine
Lamivudine
Leflunomide
Levofloxacin
Losartan
Methotrexate
Mitiglinide
Naftopidil
Pioglitazone
Pranlukast
Pravastatin
Ranitidine
Rosuvastatin
Sertraline
Simvastatin
Tacrolimus
Ticlopidine
Tranilast
Ursodeoxycholic acid
Valproate
Valsartan
Voriconazole
B
SCHH (Hu1437) toxicity (%)
0
50
100
Amiodarone
Atorvastatin
Carbamazepine
Clopidogrel
Cyclosporine A
Everolimus
Famotidine
Fexofenadine
Flutamide
Lamivudine
Leflunomide
Levofloxacin
Losartan
Methotrexate
Pioglitazone
Pravastatin
Rosuvastatin
Simvastatin
Tacrolimus
Ticlopidine
Tranilast
Voriconazole
C
SCHH (Hu1524) toxicity (%)
0
50
100
Amiodarone
Atorvastatin
Carbamazepine
Clopidogrel
Cyclosporine A
Everolimus
Famotidine
Fexofenadine
Flutamide
Lamivudine
Leflunomide
Levofloxacin
Losartan
Methotrexate
Pioglitazone
Pravastatin
Rosuvastatin
Simvastatin
Tacrolimus
Ticlopidine
Tranilast
Voriconazole
D
SCHH (Hu4197) toxicity (%)
0
50
100
Amiodarone
Atorvastatin
Carbamazepine
Clopidogrel
Cyclosporine A
Everolimus
Famotidine
Fexofenadine
Flutamide
Lamivudine
Leflunomide
Levofloxacin
Losartan
Methotrexate
Pioglitazone
Pravastatin
Rosuvastatin
Simvastatin
Tacrolimus
Ticlopidine
Tranilast
Voriconazole

Fig. 4

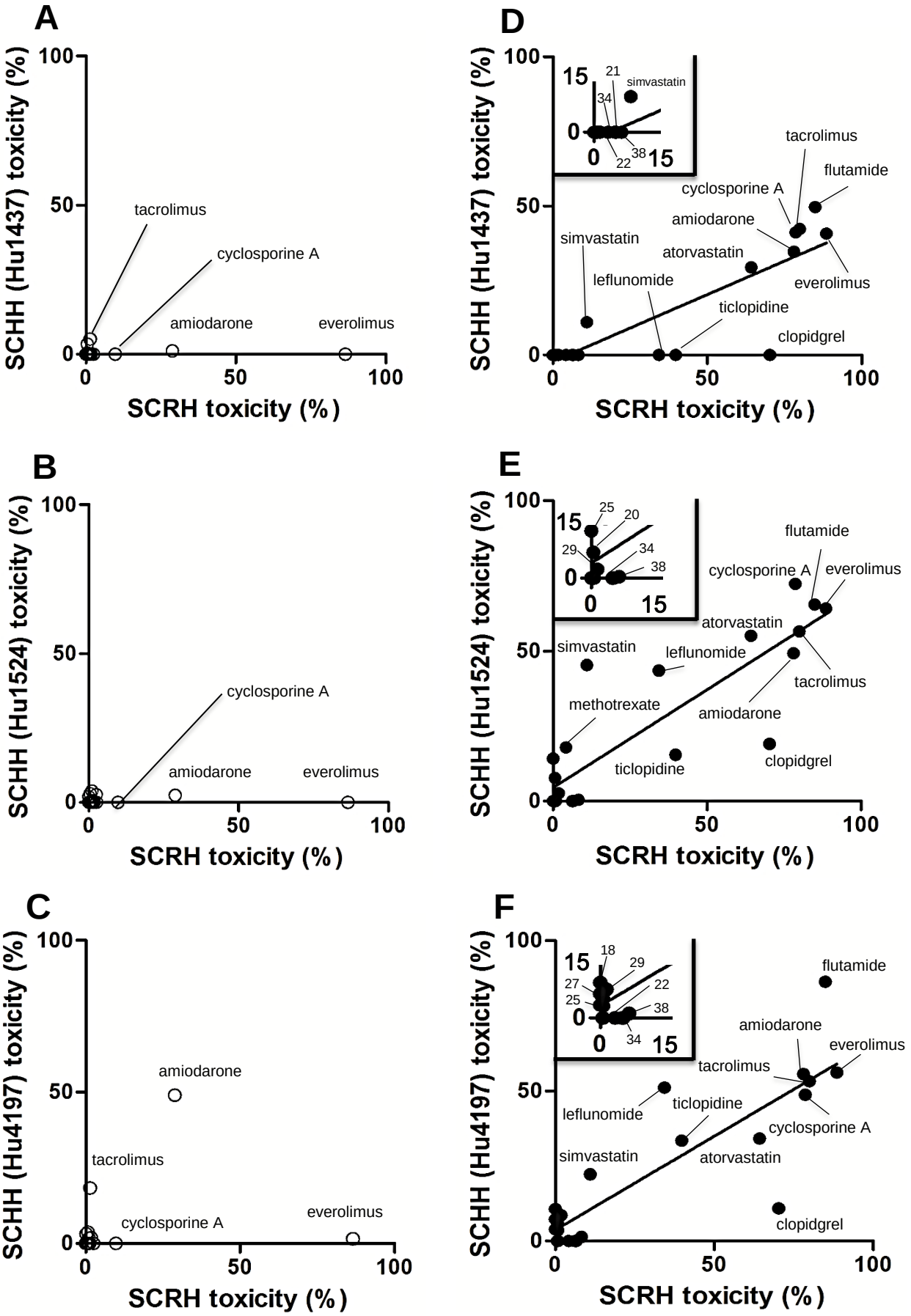
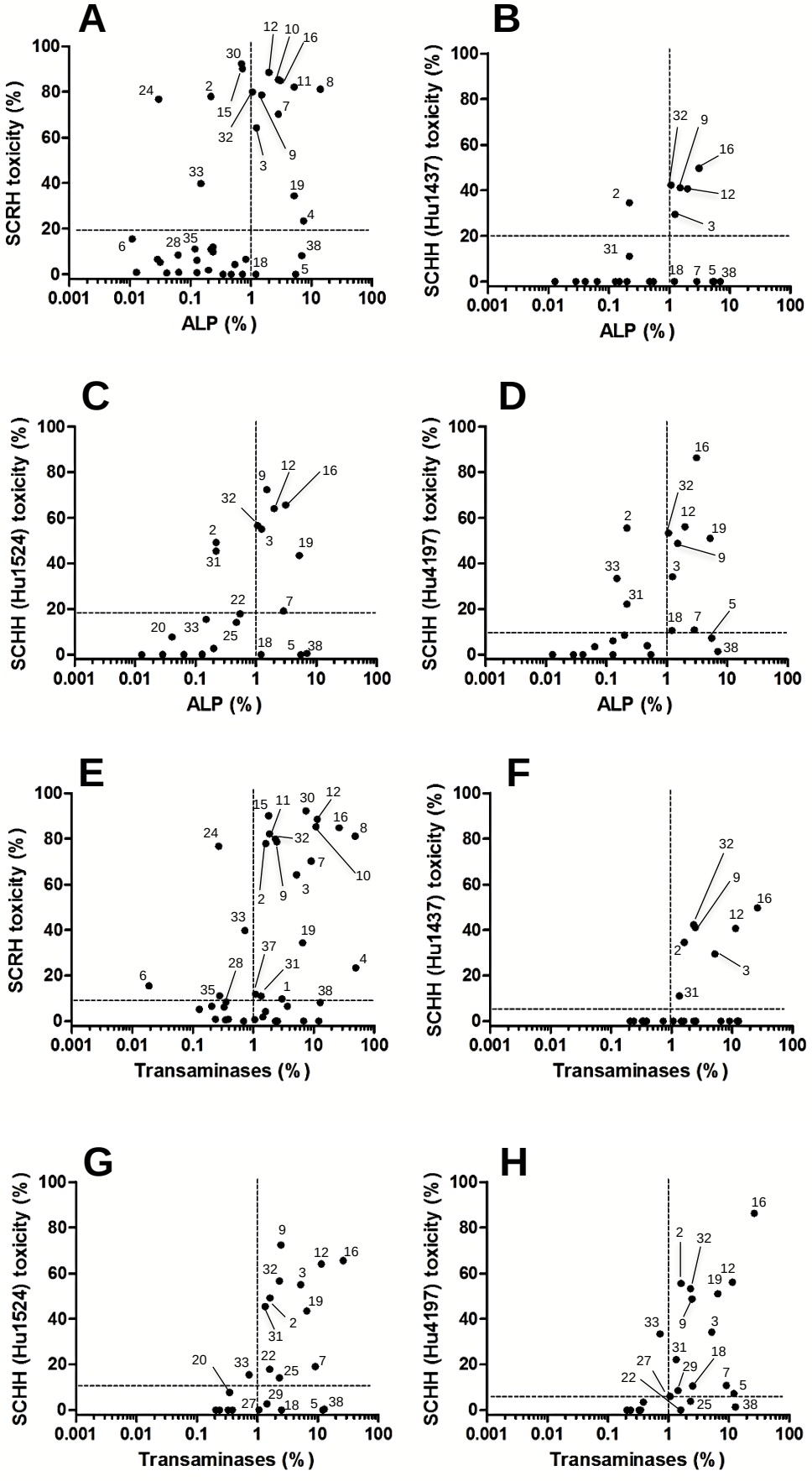

A
SCHH (Hu1437) toxicity (%)
SCRH toxicity (%)
tacrolimus
cyclosporine A
amiodarone
everolimus
B
SCHH (Hu1524) toxicity (%)
SCRH toxicity (%)
cyclosporine A
amiodarone
everolimus
C
SCHH (Hu4197) toxicity (%)
SCRH toxicity (%)
amiodarone
tacrolimus
cyclosporine A
everolimus
D
SCHH (Hu1437) toxicity (%)
SCRH toxicity (%)
simvastatin
tacrolimus
flutamide
cyclosporine A
amiodarone
atorvastatin
everolimus
leflunomide
ticlopidine
clopidgrel
E
SCHH (Hu1524) toxicity (%)
SCRH toxicity (%)
flutamide
cyclosporine A
everolimus
atorvastatin
simvastatin
leflunomide
tacrolimus
methotrexate
amiodarone
ticlopidine
clopidgrel
F
SCHH (Hu4197) toxicity (%)
SCRH toxicity (%)
flutamide
amiodarone
everolimus
tacrolimus
leflunomide
ticlopidine
cyclosporine A
simvastatin
atorvastatin
clopidgrel

Fig. 5


A
SCRH toxicity (%)
ALP (%)
B
SCHH (Hu1437) toxicity (%)
ALP (%)
C
SCHH (Hu1524) toxicity (%)
ALP (%)
D
SCHH (Hu4197) toxicity (%)
ALP (%)
E
SCRH toxicity (%)
Transaminases (%)
F
SCHH (Hu1437) toxicity (%)
Transaminases (%)
G
SCHH (Hu1524) toxicity (%)
Transaminases (%)
H
SCHH (Hu4197) toxicity (%)
Transaminases (%)