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Drug-induced cholestasis risk assessment in sandwich-cultured human hepatocytes

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ABSTRACT

Drug-induced cholestasis (DIC) is recognized as one of the prime mechanisms for DILI. Hence, earlier detection of drug candidates with cholestatic signature is crucial. Recently, we introduced an *in vitro* model for DIC and evaluated its performance with several cholestatic drugs. We presently expand on the validation of this model by 14 training compounds (TCs) of the EU-EFPIA IMI project MIP-DILI.

Several batches of human hepatocytes in sandwich-culture were qualified for DIC assessment by verifying the bile acid-dependent increase in sensitivity to the toxic effects of cyclosporin A. The cholestatic potential of the TCs was expressed by determining the drug-induced cholestasis index (DICI). A safety margin (SM) was calculated as the ratio of the lowest TC concentration with a DICI ≤ 0.80 to the $C_{max,total}$. Nefazodone, bosentan, perhexiline and troglitazone were flagged for cholestasis (SM < 30). The hepatotoxic (but non-cholestatic) compounds, amiodarone, diclofenac, fialuridine and ximelagatran, and all non-hepatotoxic compounds were cleared as "safe" for DIC. Tolcapone and paracetamol yielded DICI-based SM values equal to or higher than those based on cytotoxicity, thus excluding DIC as a DILI mechanism.

This hepatocyte-based *in vitro* assay provides a unique tool for early and reliable identification of drug candidates with cholestasis risk.

1. Introduction

Drug-induced liver injury (DILI) plays a substantial role in clinical trial failures and post-marketing withdrawals. Symptoms of cholestasis occur in about 50% of the DILI cases reported in literature (Lee, 2003). According to Fischer et al., cholestasis is a pathological condition in which the bile secretion and flow is impaired, leading to hepatocytic accumulation of bile acids (BAs) and other cholephiles (Fischer et al., 1996). The subsequent toxicity, exerted by the accumulated BAs, is due to the fact that intracellular BAs can cause a disruption of the mitochondrial ATP synthesis, resulting in apoptosis or necrosis (Maillette de BuyWenniger and Beuers, 2010; Perez, 2009). The alleged role of BA homeostasis disturbances in different forms of hepatotoxicity is consistent with *in vivo* observations and *in vitro* data (Chatterjee et al., 2014a; Yamazaki et al., 2013).

The BA homeostasis is tightly regulated and requires the multiplex interplay between different transporters and metabolic enzymes in both liver and intestine. In hepatocytes, BAs are actively taken up by the sinusoidal uptake transporters, namely the sodium taurocholate co-transporting polypeptide (NTCP; *SLC10A1*) and the members of the organic anion transporting polypeptide (OATP; *SLCO* family). The excretion into bile canaliculi is facilitated primarily by the bile salt export pump (BSEP; *ABCB11*). Additionally, sulfated and glucuronidated BAs are transported by the canalicular efflux transporter multidrug resistance-associated protein 2 (MRP2; *ABCC2*) (Pauli-Magnus and Meier, 2006). The biliary excretion of cholesterol, which is a precursor of BAs, is facilitated by the heterodimer ABCG5/ABCG8, located in the canalicular membrane of the hepatocytes (Yu et al., 2004). At the

Abbreviations: BAs, bile acids; C_{max}, maximum plasma concentration; CDF(DA), 5-(6)-carboxy-2′,7′-dichlorofluorescein (diacetate); DIC(I), drug-induced cholestasis (index); DILI, drug-induced liver injury; IMI, Innovative Medicine Initiative; MIP-DILI, Mechanism-Based Integrated Systems for the Prediction of Drug-Induced Liver Injury; RMF, reactive metabolite formation; SC(H)H, sandwich-cultured (human) hepatocytes; SM, safety margin; TC, training compound.

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sinusoidal membrane, BAs are transported back into the blood by the heterodimeric organic solute transporter (OSTα/OSTβ) (Ballatori et al., 2005). Under cholestatic conditions, the sinusoidal efflux transporters, multidrug resistance-associated protein 3 and 4 (MRP3, *ABCC3*; MRP4, *ABCC4*) are upregulated as a protective mechanism to transport BAs back into the blood (Alrefai and Gill, 2007; Bohan and Boyer, 2002).

BSEP inhibition has been implicated as the major mechanism of drug-induced cholestasis (DIC) (Kubitz et al., 2012). Examples of currently existing *in vitro* models to detect DIC rely on the inhibition of BSEP-mediated taurocholic acid transport, either in sandwich-cultured hepatocytes (SCH) or in BSEP overexpressing membrane vesicles (Dawson et al., 2012; Marion et al., 2007; Morgan et al., 2010; Pedersen et al., 2013). However, several recent studies support the recognition that evaluation of BSEP inhibition alone does not sufficiently predict DIC. Indeed other mechanisms (*e.g.* inhibition of other BA transporters) or an interplay between different mechanisms are involved (Chatterjee et al., 2014b; Fukuda et al., 2013). More specifically, it has been shown that inhibition of MRP4, next to BSEP inhibition, is associated with DIC (Kock et al., 2013). In that respect, sandwich-cultured human hepatocytes (SCHH) represent a suitable *in vitro* model to investigate the effect of xenobiotics on toxicity caused by accumulating BAs subsequent to disturbed BA disposition. Previous work has indicated that SCHH preserve the disposition pathways and cellular functions involved in hepatocytic BA handling (De Bruyn et al., 2013).

Recently, we successfully introduced and applied an *in vitro* model based on SCHH relying on the widely recognized mechanism of intracellular BA accumulation associated with DIC. Essentially, the *in vitro* cholestatic potential of the training compounds (TCs) was expressed by determining drug-induced cholestasis index (DICI) values. The DICI value reflects the relative residual urea formation by hepatocytes co-incubated with a TC at a specific concentration and BAs as compared to hepatocytes treated with TC alone. The ability of this model to identify compounds that may cause cholestasis in human by interfering with BA disposition was illustrated by a correlation between clinical incidence data on cholestasis on the one hand, and an *in vitro* DICI-based safety margin (SM) on the other hand. This SM was obtained as the lowest incubation concentration (μM) yielding a DICI ≤ 0.80, divided by the total therapeutic peak plasma concentration ($C_{max,total}$, μM) in human (Chatterjee et al., 2014b).

We presently expand on demonstrating the utility and robustness of this *in vitro* model for early detection of drug candidates with risk for cholestasis by retrospectively evaluating a total of 14 TCs (Table 1 and Supplemental Table 2) of the EU-EFPIA Innovative Medicine Initiative (IMI) project "Mechanism-Based Integrated Systems for the Prediction of Drug-Induced Liver Injury (MIP-DILI)". For the purpose of the present study, these 14 TCs were first classified according to the presence or the absence of clinical evidence on DIC and DILI (see Table 1).

2. Materials and methods

The study design applied/used for determination of the DICI values is depicted in Fig. 1.

2.1. Biliary excretory function assessment by light and fluorescence microscopic imaging

The biliary excretory function in SCHH was assessed as described by Oorts et al., 2015. The procedure relies on the qualitative evaluation of 5-(6)-carboxy-2′,7′-dichlorofluorescein (CDF) excretion in biliary networks *via* fluorescence microscopy (ex 490 nm; em 520 nm). Light microscopic images of the SCHH were taken daily. Representative light microscopic and fluorescent images of each batch used during the study are shown in Supplemental Fig. 1.

2.2. Incubation with TC and BAs

A fifty-fold concentrated solution of a BA mixture consisting of the five quantitatively most important BAs present in human plasma, was used (see Table 2; Chatterjee et al., 2014b). Hepatocytes were first incubated with the TC alone for 2 h, to provide the TC with the time to interfere with the hepatic transporters in the absence of potentially competing BAs. Subsequently, the incubation medium was replaced by a mixture of the TC (at the same concentration) and BAs, and incubated for another 22 h. After the incubations, a urea assay was performed for a quantitative assessment of (compromised) hepatocyte functionality. Furthermore, SCHH were re-exposed a second time for 24 h to the TC and BAs, followed by another urea assay, to assess the *in vitro* cholestatic effect of repeated exposure.

Table 1
Classification of the set of 14 training compounds (TCs) of the EU-EFPIA Innovative Medicine Initiative (IMI) project "Mechanism-Based Integrated Systems for the Prediction of Drug-Induced Liver Injury (MIP-DILI)". Class A represents TCs with known reports on drug-induced liver injury (DILI) *in vivo*, while those in Class B are TCs with reports on drug-induced cholestasis (DIC) *in vivo*. The mechanism of toxicity based on literature data is also provided. Class C compounds are generally considered safe regarding DILI.

Classes	Compounds	Mechanism of toxicity	References
Class A	Acetaminophen	RMF[a]; mitochondrial toxicity	(Kaplowitz, 2013; Larson, 2007)
	Amiodarone	Phospholipidosis; steatosis; mitochondrial toxicity (inhibition of mitochondrial β-oxidation)	(Flaharty et al., 1989; Fromenty and Pessayre, 1995)
	Diclofenac	RMF; oxidative stress; mitochondrial toxicity; inflammatory-immune reactions	(Boelsterli, 2003; Unzueta and Vargas, 2013)
	Tolcapone	Mitochondrial toxicity	(Dykens and Will, 2007; Olanow, 2000)
	Ximelagatran	Mechanism is not fully elucidated yet; changes in plasma membrane properties	(Kenne et al., 2008; Lee et al., 2005; Sergent et al., 2009)
	Perhexiline	Steatohepatitis; RMF; phospholipidosis; mitochondrial toxicity (inhibition of mitochondrial β-oxidation and respiratory chain)	(Ashrafian et al., 2007; Fromenty and Pessayre, 1995)
	Fialuridine	Mitochondrial dysfunction; steatosis	(Kleiner et al., 1997; Xu et al., 2014)
Class B	Cyclosporin A	Cholestasis (inhibition of bile acid transport; BSEP inhibition); oxidative stress; Mitochondrial toxicity	(Kaplowitz, 2013; Kis et al., 2012; Padda et al., 2011; Van den Hof et al., 2014)
	Troglitazone	Cholestasis (inhibition of bile acid transport; BSEP inhibition); mitochondrial toxicity; RMF; oxidative stress	(Kaplowitz, 2013; Lebovitz et al., 2002)
	Nefazodone	RMF; mitochondrial toxicity; cholestasis (inhibition of bile acid transport; BSEP inhibition)	(Dykens et al., 2008; Kostrubsky et al., 2006)
	Bosentan	Cholestasis: BSEP inhibition	(Fattinger et al., 2001; Kaplowitz, 2013)
Class C	Entacapone	(Unknown)/not applicable	(Fisher et al., 2002)
	Metformin	(Unknown)/not applicable	(Brackett 2010; Miralles-Linares et al., 2012)
	Buspirone	(Unknown)/not applicable	(Kostrubsky et al., 2006)
	Pioglitazone	(Unknown)/not applicable	(Lebovitz et al., 2002)

[a] RMF = reactive metabolite formation.

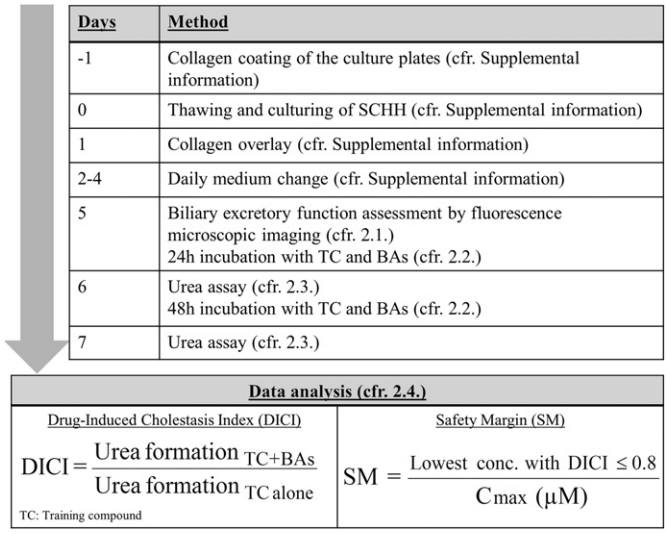

Fig. 1. Schematic overview of the drug-induced cholestasis index (DICI) assessment in sandwich-cultured human hepatocytes (SCHH).

2.3. Determination of urea production

The capacity of the hepatocytes to convert ammonia to urea was used to assess the overall biochemical function and integrity of human hepatocytes. Urea production by SCHH was determined as described previously (Chatterjee et al., 2014b). Absorbance was measured at 525 nm using a Tecan Infinite M200 plate reader (Tecan Group Ltd., Männedorf, Austria).

2.4. Data analysis

To quantify the ability of a TC to exert toxicity by disturbing BA homeostasis *in vitro*, a DICI value was calculated by equation 1 (Chatterjee et al., 2014b).

$$\text{DICI} = \frac{\text{Urea production in presence of TC + BAs}}{\text{Urea production in presence of TC alone}} \quad (1)$$

DICI values were calculated for each TC at every concentration examined. In case of complete loss of urea production (<40%) for incubations with the TC alone, no DICI values were calculated. DICI values were interpreted as follows: (i) mild or no potential disturbance of the *in vitro* BA homeostasis: DICI > 0.80; (ii) moderate disturbance of *in vitro* BA homeostasis: 0.80 ≥ DICI > 0.50; and (iii) pronounced disturbance of *in vitro* BA homeostasis: DICI ≤ 0.50.

DICI values ≤ 0.80 were used as a threshold for flagging a TC positive for cholestasis risk at a given concentration. In order to translate *in vitro* DICI values into *in vivo* cholestasis risk, safety margins for drug-induced cholestasis (DICI-based SM) were calculated for each TC, according to Equation 2. The calculation for DICI-based SM is based on the lowest *in vitro* concentration (μM) yielding a DICI ≤ 0.80 and the $C_{max,total}$ (μM; total therapeutic peak plasma concentration obtained from literature). The $C_{max,total}$ and $C_{max,unbound}$ of all the TCs used during this study are listed in Table 3.

$$\text{DICI-based SM} = \frac{\text{Lowest concentration (μM) yielding a DICI} \le 0.80}{\text{Cmax, total (μM)}} \quad (2)$$

When the DICI value was higher than 0.80 for the highest concentration tested, a minimum SM was reported [SM > highest concentration tested (μM) / $C_{max,total}$ (μM)].

2.5. Statistics

Data are represented as mean ± SD. A two-tailed paired Student's t-test in Microsoft Excel (version 2010) was used to evaluate statistical significance of differences between urea production (nmol/cm^2) in SCHH incubated with or without BAs. Differences were considered statistically significant when $p < 0.05$.

3. Results

3.1. Proof-of-concept data

3.1.1. Effect of the BA mixture on the biochemical functionality of SCHH

A 50-fold concentrated BA mixture was used to co-incubate the SCHH with TCs. Therefore, the effect of this mixture on the capability of the hepatocytes to convert ammonia into urea was measured. In Table 4, the mean (±SD) urea formation of 6 different human batches used throughout this study, when incubated with or without the BA mixture, is shown. It was observed that a 48 h exposure to a 50-fold concentrated BA mixture did not significantly decrease the urea produced by the hepatocytes (day-7 of culture time), compared to the control hepatocytes ($p > 0.05$). As this 50-fold concentrated BA mixture caused no toxicity, it was applied during subsequent studies.

3.1.2. Inter-donor and intra-donor variability of cyclosporin A

We previously reported that the well-known cholestatic drug cyclosporin A (Padda et al., 2011) sensitized SCHH to the cytotoxic effects of a mixture of BAs (Chatterjee et al., 2014b). In order to confirm that

Table 2
Composition of the bile acid (BA) mixture used for the drug-induced cholestasis risk assessment in sandwich-cultured human hepatocytes (SCHH). SCHH were exposed to a 50-fold concentrated solution of a mixture of BAs, consisting of the five quantitatively most important BAs present in human plasma (Gnewuch, 2009; Scherer et al., 2009; Xiang et al., 2010).

Bile acids	Concentration in plasma [μM]	In vitro assay concentration (50×) [μM]
Chenodeoxycholic acid	0.39	19.5
Glycochenodeoxycholic acid	1.32	66
Glycodeoxycholic acid	0.38	19
Deoxycholic acid	0.40	20
Glycocholic acid	0.35	17.5
Total	2.84	142

Table 3
Reported (unbound) maximum plasma concentrations ($C_{max,total}$ and $C_{max,unbound}$) of the 14 training compounds (TCs).

Class	Compounds	$C_{max,total}$ (μM)	$C_{max,unbound}$ (μM)	References
Class A	Acetaminophen	139	139	(Xu et al., 2008)
	Amiodarone	0.81	$8.1 * 10^{-4}$	(Xu et al., 2008; Zhang et al., 2012)
	Diclofenac	7.99	0.04	(Xu et al., 2008; Zhang et al., 2012)
	Tolcapone	47.6	0.48	(Dawson et al., 2012)
	Ximelagatran	0.30	0.26	(Francis, 2004; Keisu and Andersson, 2010)
	Perhexiline	2.16	0.22	(Xu et al., 2008)
	Fialuridine	0.64	0.23	(Dawson et al., 2012)
Class B	Cyclosporin A	0.77	0.05	(Dawson et al., 2012; Xu et al., 2008)
	Troglitazone	6.39	0.06	(Dawson et al., 2012; Xu et al., 2008)
	Nefazodone	4.26	0.04	(Dawson et al., 2012)
	Bosentan	7.39	0.15	(Dawson et al., 2012)
Class C	Entacapone	4.34	0.09	(Rouru et al., 1999)
	Metformin	7.75	7.5	(Yang et al., 2013)
	Buspirone	0.01	$5.0 * 10^{-4}$	(Dawson et al., 2012)
	Pioglitazone	2.95	0.03	(Dawson et al., 2012; Xu et al., 2008)

Table 4
Comparison of the mean (±SD) urea formation in day-7 sandwich-cultured human hepatocytes (SCHH), incubated for 48 h with or without the bile acid (BA) mixture. No significant differences were seen between the 2 conditions as compared by the Student's t-test ($p > 0.05$).

Donor	Urea formation (−BAs) (nmol/cm^2)	Urea formation (+BAs) (nmol/cm^2)
S1093T	22.78 ± 0.43	25.25 ± 0.83
L090109	9.63 ± 1.39	11.62 ± 2.51
S1142T	16.10 ± 0.34	15.52 ± 4.12
S1026T	15.40 ± 0.36	15.50 ± 1.29
S1236T	14.06 ± 2.48	13.41 ± 2.01
S1245T	18.56 ± 0.09	19.06 ± 1.23

cyclosporin A can be consistently used as a positive control in SCHH for the DIC assessment, the DICI values of 15 μM cyclosporin A obtained in various batches of SCHH after both 24 h and 48 h co-exposure to a BA mixture were determined (Fig. 2). Overall, 15 μM cyclosporin A yielded a DICI value lower than or equal to 0.80 in 11 out of 21 tested SCHH cultures after 24 h of co-exposure with BAs, while a DICI value lower than or equal to 0.80 was observed in 15 out of 18 tested SCHH cultures after 48 h of co-exposure with BAs, showing a substantial inter-donor variability.

To determine also intra-donor variation, 4 human batches were cultured on two different occasions and analyzed in 2 independent experiments. Subsequently, these independently established sandwich-cultures of human hepatocytes were also exposed to cyclosporin A (15 μM) in the presence or the absence of BAs for 24 and 48 h. As shown in Table 5, the mean (±SD) DICI values of both of the experiments per batch were similar and classified cyclosporin A in a comparable way, meaning that intra-donor variability is low as compared to inter-donor variability.

The reduction in DICI values was found to be more pronounced when SCHH were exposed for a second 24 h period to 15 μM cyclosporin A and BAs. Overall, the mean DICI value, after 24 and 48 h yielded 0.71 and 0.59, respectively. For further studies, "cyclosporin A-DICI-positive" (DICI ≤ 0.80) human hepatocyte donor batches were used.

3.2. DICI assessment of the MIP-DILI set of 14 TCs

After confirming the presence of functional bile canaliculi at day-5 of culture time (see Supplemental Fig. 1), several batches of human hepatocytes were selected based on their sensitivity to the *in vitro* cholestatic effect of cyclosporin A. These batches were used to assess changes in urea production upon 24 h or 48 h co-exposure to various TCs and BAs. Table 6 represents the mean (±SD) DICI values of the entire MIP-DILI set of TCs.

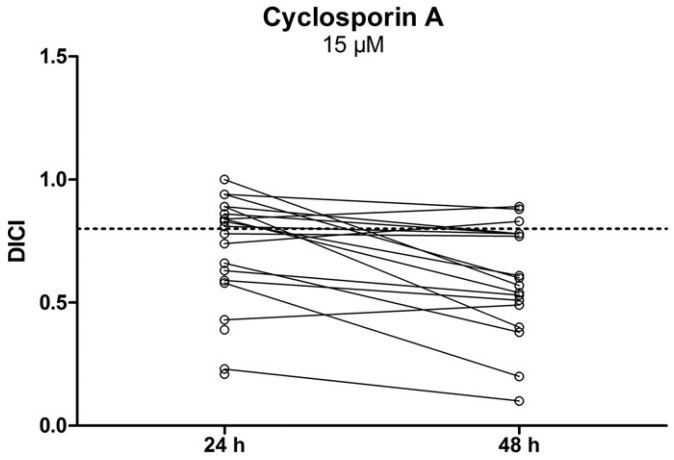

Fig. 2. Drug-induced cholestasis index (DICI) of cyclosporin A in sandwich-cultured human hepatocytes (SCHH). SCHH (n = 21 individual donors) were incubated with 15 μM cyclosporin A in the presence or the absence of bile acids (BAs) for a single (24 h) and a repeated (48 h) exposure to obtain DICI values (in triplicate wells). Each point represents a DICI value obtained in one batch of SCHH. Connecting lines show DICI values obtained in the same batch of SCHH. The dotted line on the Y-axis represents a DICI value of 0.80.

Table 5
Intra-donor variability of mean (±SD) drug-induced cholestasis index (DICI) values of cyclosporin A (15 μM) after a single exposure (24 h) and after repeated exposure (48 h) in different batches (n = 4) of sandwich-cultured human hepatocytes (SCHH). SCHH were seeded and exposed to cyclosporin A and/or bile acids (BAs) at different occasions (i.e. independent experiments were performed with each individual batch). Values marked in gray indicate measurements where cyclosporin A exerted a disturbance in BA homeostasis.

	Cyclosporin A (15 μM)	
Human donor batch	DICI ± SD after 24 h	DICI ± SD after 48 h
S1236T	0.83 ± 0.26	0.61 ± 0.24
	0.95 ± 0.24	0.70 ± 0.17
S1245T	0.73 ± 0.07	0.79 ± 0.10
	0.79 ± 0.09	0.77 ± 0.10
S1026T	0.65 ± 0.26	0.38 ± 0.18
	0.66 ± 0.11	0.33 ± 0.18
S1142T	0.98 ± 0.15	0.75 ± 0.24
	0.89 ± 0.20	0.40 ± 0.20

The Class A compounds did not show DICI values lower than 0.80 after 24 h and 48 h of exposure at their highest tested concentrations. However, ximelagatran yielded a (borderline) DICI (±SD) value of 0.79 ± 0.22 after incubating the TC at 25 μM (the lowest tested concentration) for 48 h. Nonetheless, higher concentrations (up to 150 μM) of ximelagatran did not show any decrease in urea production with or without BAs (no concentration-dependent effect). Perhexiline (<9 μM) did not show any decrease in urea formation in the presence or the absence of BAs after 24 h, while after 48 h of exposure time, a DICI (±SD) value of 0.80 ± 0.09 was observed. At a higher tested concentration (65 μM), perhexiline was cytotoxic to the SCHH, even when the TC was incubated alone. Fialuridine (10–20 μM) did not show any decrease in urea formation in the presence or the absence of BAs after 24 and 48 h, except for 30 μM fialuridine, which yielded a (borderline) DICI (±SD) value of 0.79 ± 0.16.

The Class B (*i.e.* cholestatic) compounds, troglitazone and nefazodone, displayed marked and concentration-dependent toxicity in the presence of BAs, yielding DICI values ≤ 0.80. The highest tested concentration (130 μM) of nefazodone was too cytotoxic to the SCHH, even when the TC was incubated alone. Bosentan did not decrease the urea formation in the presence of BAs in this particular batch of SCHH (S1142T). However, previous data showed that bosentan yielded a DICI value lower than 0.80 in two out of four tested batches of SCHH (Chatterjee et al., 2014b).

The Class C compounds metformin and pioglitazone did not show DICI values lower than 0.80 at their highest tested concentrations (respectively 900 μM and 300 μM). Buspirone, at 0.5 and 5 μM after 24 h of exposure showed DICI (±SD) values of 0.69 ± 0.50 and 0.74 ± 0.53, respectively. However, after 48 h of exposure time, DICI (±SD) values were higher than 0.80, suggesting recovery of cultures from disturbed BA homeostasis. Entacapone induced a decrease in urea formation in the presence of BAs at its highest concentration tested (130 μM), yielding a DICI (±SD) of 0.62 ± 0.07 and 0.10 ± 0.03, after 24 and 48 h of exposure, respectively.

3.3. Interpretation of the DICI-based SM

Based on the $C_{max,total}$ of each TC (see Table 3), the DICI-based SMs were calculated with equation 2 and listed in Table 7. TCs for which a DICI-based SM of at least 30 could not be verified included:

Table 6
Mean (±SD) drug-induced cholestasis index (DICI) values of the 14 MIP-DILI training compounds (TCs) after a single exposure (24 h) and after repeated exposure (48 h) of a selected batch of sandwich-cultured human hepatocytes (SCHH). Values in gray show a disturbance in bile acid homeostasis (DICI ≤0.80).

Compound	Concentration (μM)	DICI 24 h ± SD	DICI 48 h ± SD
Acetaminophen	200	0.92 ± 0.07	0.96 ± 0.11
	500	0.91 ± 0.09	1.02 ± 0.07
	1000	0.98 ± 0.03	1.02 ± 0.01
Amiodarone	5	0.97 ± 0.02	1.03 ± 0.17
	10	1.09 ± 0.16	1.10 ± 0.16
	25	0.96 ± 0.16	0.99 ± 0.07
Diclofenac	100	1.18 ± 0.16	0.95 ± 0.20
	300	1.34 ± 0.30	1.19 ± 0.23
	500	1.16 ± 0.24	1.04 ± 0.19
Tolcapone	5	0.98 ± 0.18	1.00 ± 0.19
	10	1.05 ± 0.16	1.01 ± 0.06
	50	0.91 ± 0.23	0.89 ± 0.24
Ximelagatran	25	0.85 ± 0.17	0.79 ± 0.22
	50	0.95 ± 0.14	0.95 ± 0.21
	150	1.02 ± 0.07	0.93 ± 0.09
Perhexiline	3	1.00 ± 0.11	1.05 ± 0.09
	9	1.03 ± 0.18	0.80 ± 0.09
	65	*n.c.*	*n.c.*
Fialuridine	10	0.88 ± 0.31	1.02 ± 0.07
	20	0.94 ± 0.35	0.92 ± 0.16
	30	0.95 ± 0.39	0.79 ± 0.16
Troglitazone	20	0.98 ± 0.09	1.09 ± 0.10
	50	1.02 ± 0.22	1.00 ± 0.10
	100	0.79 ± 0.01	0.37 ± 0.27
Nefazodone	10	0.69 ± 0.11	1.18 ± 0.37
	30	0.77 ± 0.15	0.37 ± 0.32
	130	*n.c.*	*n.c.*
Bosentan	50	1.07 ± 0.10	1.06 ± 0.13
	100	1.07 ± 0.14	1.02 ± 0.15
	200	1.05 ± 0.15	0.83 ± 0.14
Entacapone	10	1.11 ± 0.19	1.02 ± 0.33
	30	1.07 ± 0.11	0.98 ± 0.11
	130	0.62 ± 0.07	0.10 ± 0.03
Metformin	150	1.09 ± 0.30	1.34 ± 0.63
	300	1.03 ± 0.30	1.26 ± 0.62
	900	1.12 ± 0.42	1.18 ± 0.49
Buspirone	0.5	0.69 ± 0.50	0.82 ± 0.27
	1	0.87 ± 0.69	0.90 ± 0.36
	5	0.74 ± 0.53	0.95 ± 0.39
Pioglitazone	50	1.15 ± 0.09	1.06 ± 0.14
	100	1.00 ± 0.04	0.88 ± 0.05
	300	0.94 ± 0.06	0.98 ± 0.06

'*n.c.*' = *not calculated* (as the TC alone reduced urea formation by 40% or more).

acetaminophen, tolcapone, perhexiline, cyclosporin A, troglitazone, nefazodone and bosentan. Regarding acetaminophen and tolcapone, a DICI-based SM > 7 and >1 was calculated, respectively, meaning that no toxicity was observed for these TCs at the highest tested concentrations. Moreover, the SM values for DIC were equal to those obtained for general cytotoxicity (based on incubations with TC alone; Supplemental Table 3), suggesting that cholestasis was not identified as a particular mechanism for DILI. Perhexiline yielded a low SM (*i.e.* 4) for DIC in our assay, which was based on a (borderline) DICI value of 0.80 ± 0.05 at 9 μM perhexiline. Note that a potentially higher SM value could not be verified as perhexiline concentrations exceeding 9 μM were cytotoxic in the absence of BAs. This finding based on the urea assay was consistent with the cytotoxicity-based SM of 5 as assessed with the resazurin assay (see Supplemental Table 3). Bosentan yielded a SM for DIC higher than 27 with the human hepatocyte batch selected in the present study. However, we have to take into account that bosentan was evaluated previously with this DIC assay in 4 different batches, yielding a SM value below 1 in at least 1 batch (Chatterjee et al., 2014b). Based on the measurements performed in the present study, nefazodone, troglitazone and cyclosporin A were unambiguously classified for DIC risk.

Table 7
Safety margins (SMs) of the 14 training compounds (TCs) according to the $C_{max,total}$ given in Table 3 and the DICI values in Table 6. SMs obtained during this study and Chatterjee et al., 2014b are comparable. TCs with a SM for DIC < 30 are classified as compounds with a potential to cause cholestasis (Fig. 3).

Compounds	DICI-based SM, obtained in this study	DICI-based SM, obtained from Chatterjee et al. (2014b)
Acetaminophen	>7	>7
Amiodarone	>31	>25
Diclofenac	>63	>83.6
Tolcapone	>1	–
Ximelagatran	83	–
Perhexiline	4[a]	–
Fialuridine	47	–
Cyclosporin A	19	8.30
Troglitazone	16	15.6
Nefazodone	2	–
Bosentan	>27	0.14
Entacapone	30	–
Metformin	>116	–
Buspirone	50	–
Pioglitazone	>102	–

[a] "Borderline" DICI ~ 0.80.

4. Discussion

We previously developed and applied a hepatocyte-based *in vitro* model to identify drug candidates that may cause cholestasis by interfering with the BA homeostasis (Chatterjee et al., 2014b). For the purpose of *in vitro* data presentation and interpretation, the calculation of the so-called DICI was introduced. The DICI informs on the ability of a TC (incubated at a given concentration) to disturb BA homeostasis *in vitro*. In order to translate the DICI values obtained *in vitro* into risk for cholestasis *in vivo* (at therapeutically relevant concentrations), SMs are calculated. A cut-off SM value of 30 was used, implying that compounds causing a reduction in the DICI at *in vitro* concentrations exceeding peak plasma concentrations by less than 30-fold were flagged for cholestasis risk. The benefit of calculating SM values is that it provides a sensible concept for making data-driven decisions regarding the expected cholestasis risk *in vivo*. In addition, it also allows taking into account the wide interindividual variability in susceptibility to BA-mediated toxicity. This concept was also applied to assess the risk for drug-induced cardiotoxicity in the clinic based on *in vitro* data (Yao et al., 2008).

We have presently applied this hepatocyte-based *in vitro* model for DIC to investigate the potential of an additional set of TCs to cause DIC. The set of 14 TCs has been selected by the MIP-DILI consortium.

The DIC risk assessment of the TC set was preceded with investigation of the effect of the BAs themselves on the urea formation of the hepatocytes. Our findings indicate that a 50-fold concentrated mixture of these BAs (in total 142 μM BAs; Table 2) did not significantly decrease the functionality of the human hepatocyte batches (n = 6) on day-7 of culture time for minimum 48 h (Table 4). Hence, the toxicity observed when a cholestatic compound is co-incubated with BAs is solely due to the drug-induced disturbance of the BA homeostasis and not due to toxicity of the BAs themselves.

Subsequently, the inter- and intra-donor variability of the well-known cholestatic drug cyclosporin A (Padda et al., 2011) was examined. Indeed, careful selection of hepatocyte batches that are sufficiently sensitive to the toxic effects of known cholestatic compounds is key to establishing an *in vitro* platform for DIC that does not require an unacceptably high number of different batches to be tested. This holds true especially given the clinical DIC incidence rates that are usually less than 10%. Thus, combining the use of DIC-qualified batches (in the present study based on cyclosporin A) with the SM concept meets a balance of the criteria including sensible use of hepatocytes on the one hand and reliable predictions on the other hand.

Based on previous experience, a cyclosporin A concentration of 15 μM was applied to explore inter- and intrabatch variabilities. We presently confirm that the SCHH-based *in vitro* assay consistently identifies cyclosporin A as a compound that causes cholestasis by interfering with BA disposition. A pronounced inter-donor variability in cyclosporin A-induced cholestasis was shown *in vitro* with DICI values ranging between 0.10 and 1.00 (Fig. 2). This variable DIC response *in vitro* may be partially

attributed to the large inter-individual variability encountered with DILI in patients. Intra-individual variability of the human hepatocyte batches was rather low, as reflected by the DICI values of cyclosporin A in multiple batches at 2 time points (Table 5). Hence, cyclosporin A was confirmed as an excellent model drug to be used as a positive control in this *in vitro* assay, its DICI (at 15 μM) being <0.80 in 90% (19/21) of SCHH cultures from various donors.

The human hepatocyte batches that tested positive for cyclosporin A-induced cholestasis, so-called "cyclosporin A DICI-positive" human donor batches, were subsequently used to assess the TCs for DIC risk. TCs were first classified based on *in vivo* reports on DILI/DIC into 3 different classes. Our starting hypothesis was that assessment of each TC in a qualified (*i.e.* DICI-positive) batch of human hepatocytes would support reliable discrimination between cholestatic compounds (Class B) and negative controls (Class A or C).

All but one (perhexiline) of the TCs in Class A were cleared (*i.e.* SM ≥ 30) for DIC risk based on *in vitro* data obtained in the present study. Perhexiline yielded a DICI-based SM similar to its cytotoxicity-based SM (estimated based on *in vitro* cytotoxicity data), strongly suggesting that cholestasis is not a major (or sole) mechanism for cholestasis *in vivo*. This is in line with literature reports of hepatocellular toxicity of perhexiline (Ashrafian et al., 2007). Tolcapone and acetaminophen corresponded to SM values of at least 1 and 7, respectively. As both of the TCs have (very) high $C_{max,total}$ values (139 μM for acetaminophen and 47.6 μM for tolcapone), DICI values were greater than 0.80 at the highest concentrations that could be tested. Moreover, the SM based on cytotoxicity of the TCs alone were equal (and likely lower) than the DIC SM value, largely discounting a cholestatic mechanism in their DILI effect (Supplemental Table 3). Note that both TCs are reported in literature to cause severe liver damage (Borges, 2005; Bunchorntavakul and Reddy, 2013; McGill et al., 2012; Watkins, 2000). Ximelagatran and fialuridine (with a borderline DICI of 0.79 ± 0.22 and 0.79 ± 0.16, respectively) both yielded SM values > 30, clearing them from major cholestasis concern. Fialuridine yielded high SM values in the present study, both for DIC and general cytotoxicity. However, fialuridine has been found to cause ATP depletion (data not shown). Consistently, fialuridine caused acute liver failure during clinical trials (7 out of 15 patients treated with fialuridine developed acute liver injury, of which 5 died, during clinical trials), which was not observed in preclinical toxicology studies. The damaged liver tissue revealed marks of steatosis, cholestasis, cytoplasmic fat droplets and swollen mitochondria that contained a reduced number of cristae (Manning and Swartz, 1995; McKenzie et al., 1995). To our knowledge, this is the only report on the relationship between cholestasis and fialuridine. Mitochondrial toxicity and the appearance of intracytoplasmic lipid droplets have also been reported as mechanisms related to the hepatotoxicity of fialuridine (Kleiner et al., 1997). It follows that fialuridine-induced hepatotoxicity has several underlying mechanisms.

The Class B compounds troglitazone and nefazodone, for which DIC has eventually led to market withdrawal (Funk et al., 2001; Kostrubsky et al., 2006), displayed a markedly enhanced toxicity in presence of the BAs. As a result, the DICI-based SM for troglitazone was 16, which is in accordance with Chatterjee et al., while nefazodone also yielded a SM for DIC lower than 30, unambiguously classifying both TCs as cholestatic. Bosentan, for which a safety warning has been added to the label (Dhillon and Keating, 2009; Fattinger et al., 2001; Kenna et al., 2014), presented a DICI value higher than 0.80 in donor S1142T, used in the present study. Consequently, with a DICI-based SM > 27, it could have been classified as (virtually) 'safe'. However, DICI values below 0.80 were found in two out of five tested donors (combined data in the present study and in Chatterjee et al., 2014b), indicating the idiosyncratic behavior of this TC regarding DIC.

All TCs in Class C, namely buspirone, entacapone, metformin and pioglitazone were unambiguously classified as non-cholestatic TCs. These findings are in accordance with previous data, suggesting that these TCs do not display any or low hepatotoxic events (Brackett 2010; Fisher et al., 2002; Floyd et al., 2009; Kostrubsky et al., 2006). Taken together, these results confirm our previous observations supporting the competence of the assay to differentiate between cholestatic and non-cholestatic TCs.

Troglitazone and pioglitazone both belong to the thiazolidinedione class to treat hyperglycemia in patients with diabetes type 2. Troglitazone is a known cholestatic TC *in vivo*, while pioglitazone does not have any reports on hepatotoxicity (Lebovitz et al., 2002). Consistently, our *in vitro* DIC assay correctly distinguished between both analogs, with DICI-based SM values of 16 for troglitazone and 102 for pioglitazone. The exact mechanism of troglitazone-mediated hepatotoxicity has not been fully understood yet. Reactive metabolite formation (RMF) has been described, next to interference with BA transport, in particular due to BSEP inhibition by the sulfate metabolite of troglitazone, which is a more potent inhibitor than the parent drug (Chojkier, 2005; Funk et al., 2001; Smith, 2003). In this respect, SCHH represent an advantageous *in vitro* model for hepatotoxicity evaluation, featuring the possibility to take into account the multiplex mechanisms (*e.g.* of RMF, interference with transporters, mitochondrial toxicity, etc) that are usually involved in generating a "total" toxic outcome. This is an important asset compared with other *in vitro* models, such as transporter-transfected cell lines or membrane vesicles, where only a single mechanism of hepatotoxicity can be taken into account. Moreover, SCHH excellently support this concept since enzymes and transporters involved in hepatic drug disposition *in vivo* are reasonably well maintained as compared to many other *in vitro* models including hepatocyte-like cell lines. As a consequence, intracellular drug exposure levels are expected to be comparable between SCHH and hepatocytes *in vivo*. Nefazodone and its structural analog buspirone were also investigated in this study. Nefazodone yielded a DICI value of 0.69 ± 0.11 at 10 μM (10× lower than its IC_{30} for cytotoxicity), resulting in a low DICI-based SM of 2.3. Buspirone also yielded DICI values below 0.80, even at a lower *in vitro* incubation concentration (0.5 μM). However, due to the approximately 100-fold lower systemic unbound drug levels of buspirone, a safe DICI-based SM around 50 was achieved. Consistently, nefazodone (but not buspirone) has been reported to disturb BA homeostasis, leading to severe liver injury, which in its turn has led to the withdrawal of nefazodone from the market in 2004. No clinical evidence was found for DILI/DIC caused by buspirone (Kostrubsky et al., 2006).

Based on the obtained DICI-based SM values and the clinical reports on cholestasis, a comparative classification is presented in Fig. 3. The majority of the TCs were classified unambiguously. Further experimentation with bosentan, (acetaminophen), (tolcapone) and perhexiline is warranted. This should entail testing multiple donors and incubation design. The experimental setup that was chosen during the present study involved 2 repeated exposure periods of 24 h. Also, correlation of *in vitro* endpoints for cholestasis with gene expression data as well as activity levels of proteins involved in BA mediated toxicity are of future interest. The unique advantage of human hepatocytes to reflect at least to some extent various donor phenotypes can hardly be overestimated. This aspect remains an important advantage as compared to hepatocyte-like cell lines (*e.g. HepaRG and* HepG2 cells) that are nowadays also used for the same applications.

To conclude, we thoroughly verified a previously established *in vitro* assay, based on SCHH, to identify TCs with a liability to cause cholestasis. Specific attention was given to qualifying suitable batches of human hepatocytes for *in vitro* DIC assessment. This approach avoided the use of an unreasonably high number of hepatocyte batches in view of their limited availability. The MIP-DILI training set of 14 TCs was classified according to the presence or the absence of a significant risk for cholestasis *in vivo*. We observed that our data correlated well with the reported clinical reports on DIC. The data obtained with cyclosporin A illustrate the possibility to get insight regarding interindividual variability in sensitivity to DIC. This remains a unique advantage of primary human hepatocytes as compared to any hepatocyte-like cell line. This human

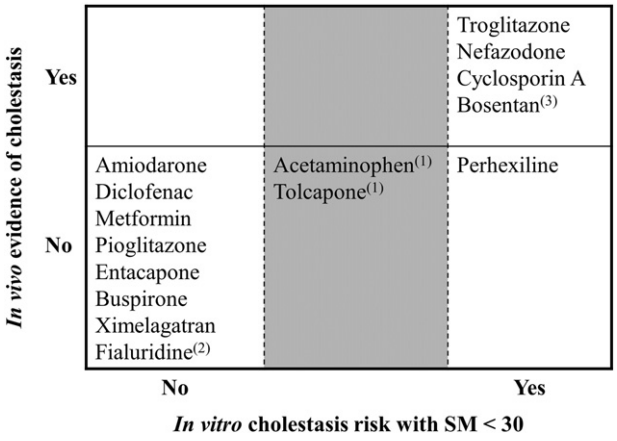

Fig. 3. Classification of all model drugs based on: (i) Literature reports of drug-induced cholestasis (DIC) *in vivo* (Y-axis); (ii) *In vitro* cholestasis risk (X-axis) of each compound as evaluated in sandwich-cultured human hepatocytes (SCHH) in the present study. Training compounds (TCs) that have clinical reports on DIC are classified in the Yes-segments (row) on the Y-axis, while TCs with no clinical reports on DIC are classified in the No-segments on the Y-axis. TCs that yielded a safety margin for DIC (DICI-based SM) < 30 are classified in the Yes-segments on the X-axis, while TCs with a DICI-based SM ≥ 30 are classified in the No-segments on the X-axis. (1)In vitro cholestasis (defined as DICI < 0.8) was not observed at the highest non-cytotoxic concentration tested; (2)Limited clinical data available; (3)SM for DIC < 30 in 2/4 batches evaluated so far in this study and Chatterjee et al., 2014b.

hepatocyte-based *in vitro* model, along with the presently obtained *in vitro* data, provides an excellent platform for future elucidation of clinically relevant mechanisms underlying DILI and DIC.

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Transparency document

The Transparency document associated with this article can be found, online version.

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