

Special Section on Transporters in Drug Disposition and Pharmacokinetic Prediction

Sandwich-Cultured Hepatocytes for Mechanistic Understanding of Hepatic Disposition of Parent Drugs and Metabolites by Transporter–Enzyme Interplay[§]

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ABSTRACT

Functional interplay between transporters and drug-metabolizing enzymes is currently one of the hottest topics in the field of drug metabolism and pharmacokinetics. Uptake transporter-enzyme interplay is important to determine intrinsic hepatic clearance based on the extended clearance concept. Enzyme and efflux transporter interplay, which includes both sinusoidal (basolateral) and canalicular efflux transporters, determines the fate of metabolites formed in the liver. As sandwich-cultured hepatocytes (SCHs) maintain metabolic activities and form a canalicular network, the whole interplay between uptake and efflux transporters and drug-metabolizing enzymes can be investigated

simultaneously. In this article, we review the utility and applicability of SCHs for mechanistic understanding of hepatic disposition of both parent drugs and metabolites. In addition, the utility of SCHs for mimicking species-specific disposition of parent drugs and metabolites in vivo is described. We also review application of SCHs for clinically relevant prediction of drug-drug interactions caused by drugs and metabolites. The usefulness of mathematical modeling of hepatic disposition of parent drugs and metabolites in SCHs is described to allow a quantitative understanding of an event in vitro and to develop a more advanced model to predict in vivo disposition.

Introduction

In drug development, pharmacokinetics play a central role in understanding the relationship of exposure with efficacy and safety. Research work in drug metabolism and pharmacokinetics has focused on optimization of absorption, distribution, metabolism, and excretion properties to give an adequate pharmacokinetics profile of a new chemical entity (NCE). These efforts have significantly reduced the failure rate of projects in clinical development due to pharmacokinetics

issues from approximately 40% in the 1990s to <10% in the 2000s (Kennedy, 1997; Frank and Hargreaves, 2003). Accumulated information on drug-metabolizing enzymes and transporters has contributed significantly to advances in drug metabolism and pharmacokinetics research. We have now entered a new era, with the goal of understanding transporter-mediated disposition (Hillgren et al., 2013), determining intrinsic clearance of metabolically stable NCEs (Di and Obach, 2015), and evaluating systemic exposure of metabolites (Yu et al., 2010).

The liver is one of the key organs for these activities because it is a major clearance organ of NCEs via metabolism and biliary excretion. In addition to these two processes, uptake and efflux on the sinusoidal (basolateral) membrane of the liver are involved in hepatic intrinsic

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ABBREVIATIONS: BA, bile acid; BCRP, breast cancer resistance protein; BDG, bilirubin diglucuronide; BEI, biliary excretion index; BMG, bilirubin monoglucuronide; BSEP, bile salt export pump; CAN, candesartan; CCX, candesartan cilexetil; CDF, 5(6)-carboxy-2',7'-dichlorofluorescein; CDFDA, 5(6)-carboxy-2',7'-dichlorofluorescein diacetate; CL_{int,bile}, intrinsic biliary excretion clearance; CL_{int,bile,app}, apparent CL_{int,bile}; CL_{int,H}, hepatic intrinsic clearance; CPD-0801, 2,5-bis (5-amidino)-2-pyridyl furan; CP-I, coproporphyrin-I; CPD-0868, 2,5-bis [5-(N-methoxyamidino)-2-pyridyl] furan; CsA, cyclosporin A; DDI, drug-drug interaction; DM-4103, tolvaptan; ECM, extracellular matrix; E₂, estradiol; E17G, estradiol 17β-D-glucuronide; MPA, mycophenolic acid; MPAG, mycophenolic acid phenyl-glucuronide; MRP, multidrug resistance-associated protein; NCE, new chemical entity; NTCP, sodium taurocholate cotransporting polypeptide; OATP, organic anion-transporting polypeptide; P450, cytochrome P450; P-gp, P-glycoprotein; QTLI, quantitative time-lapse imaging; Ro 64-1056, 4-(1-hydroxy-2-methylpropan-2-yl)-N-[5-(2-hydroxyphenoxy)-2-pyrimidin-2-yl-6-(1,1,2,2-tetradeuterio-2-hydroxyethoxy)pyrimidin-4-yl]benzenesulfonamide; Ro 47-8634, 4-tert-butyl-N-[6-(2-hydroxyethoxy)-5-(2-hydroxyphenoxy)-2-(pyrimidin-2-yl)pyrimidin-4-yl]benzene-1-sulfonamide (bosentan); Ro 48-5033, 4-(2-hydroxy-1,1-dimethyl-ethyl)-N-[6-(2-hydroxy-ethoxy)-5-(2-methoxy-phenoxy)-[2,2#1]; [bipyrimidinyl-4-yl]-benzenesulfonamide; ; SCH, sandwich-cultured hepatocyte; SN-38, 7-ethyl-10-hydroxycamptothecin; tauro-nor-THCA-24-DBD, N-(24-[7-(4-N,N-dimethylaminosulfonyl-2,1,3-benzoxadiazole)]amino-3α,7α,12α-trihydroxy-27-nor-5β-cholestan-26-oyl)-2'-aminoethanesulfonate; TCA, taurocholate; UGT, UDP-glucuronosyltransferase.

clearance ($CL_{int,H}$) based on the extended clearance concept (Varma and El-Kattan, 2016), which is calculated by eq. 1.

$$CL_{int,H} = (PS_{int,active} + PS_{int,passive}) \times \frac{(CL_{int,met} + CL_{int,bile})}{(PS_{int,passive} + PS_{int,efflux} + CL_{int,met} + CL_{int,bile})} \quad (1)$$

$PS_{int,active}$, $PS_{int,passive}$, and $PS_{int,efflux}$ are intrinsic clearances via uptake transport, passive diffusion, and basolateral efflux transport, respectively. $CL_{int,met}$ and $CL_{int,bile}$ represent intrinsic metabolic and biliary excretion clearances. When $CL_{int,met} + CL_{int,bile}$ is larger than $PS_{int,passive}$ and $PS_{int,efflux}$, $CL_{int,H}$ becomes close to $PS_{int,active}$ and $PS_{int,passive}$ and is mainly determined by active uptake transporter-mediated intrinsic clearance. In contrast, under a condition where $PS_{int,passive} + PS_{int,efflux}$ is considerable to $CL_{int,met} + CL_{int,bile}$, $CL_{int,H}$ is governed by all the individual processes (Varma and El-Kattan, 2016).

Accordingly, characterization of the four processes is important to understand functional interplay between transporters and drug-metabolizing enzymes. For instance, metabolism in the liver is a major clearance route of atorvastatin, repaglinide, and saquinavir. However, these drugs are not only substrates of cytochrome P450 (P450), but also substrates of organic anion-transporting polypeptides (OATPs). In the case of such dual substrates, uptake transporter-enzyme interplay has to be considered to determine $CL_{int,H}$, and identification of the rate-determining process is important for prediction of drug disposition in vivo (Parker and Houston, 2008; Watanabe et al., 2010; Varma et al., 2013; Varma and El-Kattan, 2016) (Fig. 1).

The extended clearance concept is basic and applies to all NCEs, meaning that the equation is applicable to metabolites as well as parent drugs. The U.S. Food and Drug Administration's guidance for safety

testing of drug metabolites, called the MIST guidance and ICH-M3(R2), first issued in 2008 and 2009, respectively (CDER and CBER, 2010; CDER, 2016), have had a significant impact on drug discovery and development in pharmaceutical companies because further evaluation of circulating metabolites is required in both nonclinical species and humans. Unlike the parent drug, a metabolite is generally formed intracellularly in the liver. Therefore, there has to be greater emphasis on enzyme–efflux transporter interplay in the liver to understand hepatic disposition and systemic exposure of metabolites mechanistically (Fig. 1).

Several in vitro test systems have been established to determine enzyme- and transporter-mediated intrinsic clearance. For instance, microsomes are commonly used for determining intrinsic metabolic clearance of NCEs by P450 enzymes and UDP-glucuronosyltransferases (UGTs) (Obach, 1999; Naritomi et al., 2001; Kilford et al., 2009; Gill et al., 2012). Cell lines or *Xenopus* oocytes overexpressing drug-associated transporters are useful for investigation of the involvement and kinetics of transporters (Tamai et al., 2001; Nozawa et al., 2005; Nakakariya et al., 2008; Tzvetkov et al., 2013). Among established in vitro systems, isolated and/or cultured hepatocytes have been widely recognized as a holistic and reliable model to investigate both enzyme- and transporter-mediated intrinsic clearances and their interplay (Soars et al., 2007; Chiba et al., 2009; Di et al., 2012), although large interindividual variability of enzyme and transporter activities is observed among donors. Several forms of hepatocytes are used to investigate enzyme- and transporter-mediated hepatic disposition, including suspended and plated hepatocytes, cocultures, and three-dimensional models (Tetsuka et al., 2017), but sandwich-cultured hepatocytes (SCHs) are the focus of this article because of their unique feature of allowing evaluation of the whole interplay between uptake and efflux transporters and drug-metabolizing enzymes.

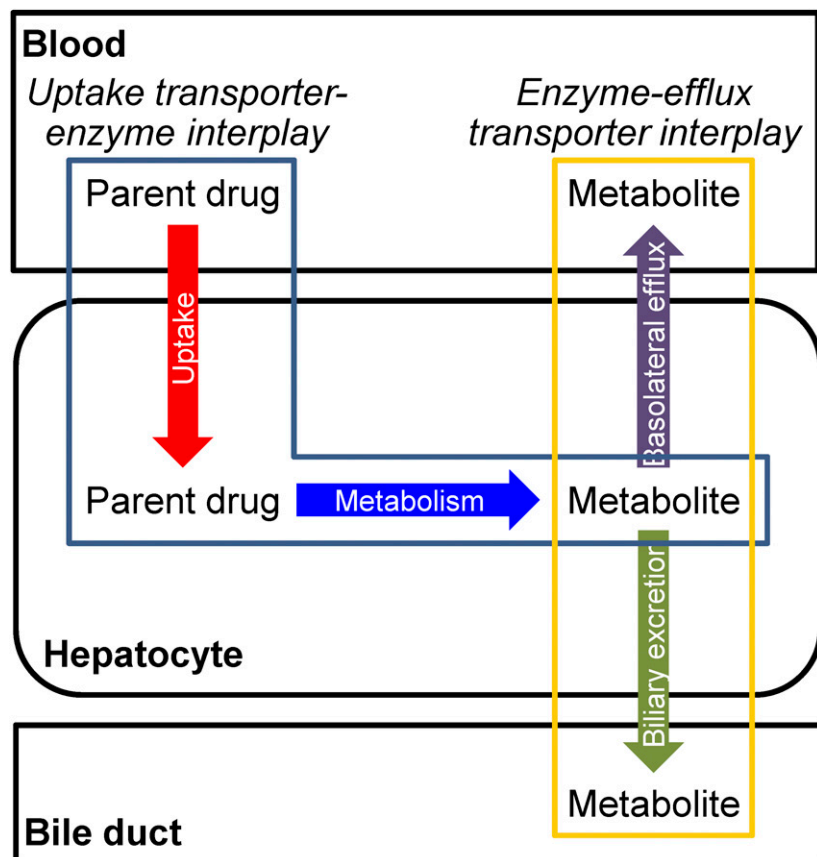


Fig. 1. Transporter–enzyme interplay in the liver.

Characteristics of SCHs

The sandwich culture system has been developed and established over two decades (Dunn et al., 1989; LeCluyse et al., 1994, 1999; Tuschl et al., 2009; Sharma et al., 2010). As the name suggests, SCHs are hepatocytes cultivated between extracellular matrices (ECMs). Commercial ECMs, Matrigel and Geltrex, are generally used to overlay hepatocytes attached to collagen I-coated plates. Compared with a conventional culture model (monolayer-plated hepatocytes), the sandwich culture system allows hepatocytes to maintain their polarity, morphology, and liver-specific activities, such as albumin secretion (Dunn et al., 1989; LeCluyse et al., 1994; Blaheta et al., 1998; Tuschl et al., 2009). There is debate about changes of metabolic enzyme activities in SCHs during culture, with some studies showing similar metabolic activities in suspended hepatocytes and SCHs (Lau et al., 2002; Treijtel et al., 2005), whereas others show lower activities in SCHs (Kern et al., 1997; Slaus et al., 2001; Kienhuis et al., 2007; Matsunaga et al., 2013). The transition of metabolic enzyme activities during culture depends on the conditions of the hepatocytes (fresh or cryopreserved) and ECM and differs among isoenzymes and species.

One of the greatest advantages of SCHs is the ability to form bile canaliculi with the entire array of transport proteins involved in biliary excretion, and the bile canaliculi networks can be disrupted by calcium/magnesium depletion in buffer (B-CLEAR Technology; Qualyst Transporter Solutions, Durham, NC) (Brouwer et al., 2013). This unique feature has allowed investigation of biliary excretion by canalicular transporters, including P-glycoprotein (P-gp), multidrug resistance-associated protein 2 (MRP2), breast cancer resistance protein (BCRP), and bile salt export pump (BSEP), in SCHs by modulation of the calcium/magnesium level in buffer.

The major parameters obtained from SCHs are the biliary excretion index (BEI) and $CL_{int,bile}$, which are calculated by eqs. 2 and 3, respectively.

$$BEI (\%) = \frac{Accumulation_{cell+bile} - Accumulation_{cell}}{Accumulation_{cell+bile}} \times 100 \quad (2)$$

$$CL_{int,bile} = \frac{Accumulation_{cell+bile} - Accumulation_{cell}}{Intracellular AUC} \quad (3)$$

BEI is a qualitative index of biliary excretion and is not correlated with in vivo extent of biliary excretion (% of dose) (Fukuda et al., 2008; Tetsuka et al., 2014a). A large interindividual variability is observed in intracellular/bile canalicular accumulations; however, BEI is relatively comparable among individual donors, especially in compounds showing high BEI (Fukuda et al., 2008; Matsunaga et al., 2014). In addition, the experiments using the same donor suggest a good reproducibility of accumulations and BEIs (Matsunaga et al., 2015, 2016).

$CL_{int,bile}$ in SCHs represents the biliary excretion clearance from hepatocytes to bile canaliculi and is correlated with the in vivo liver exposure-based $CL_{int,bile}$ (Nakakariya et al., 2012). Apparent $CL_{int,bile}$ ($CL_{int,bile,app}$) calculated from the extracellular concentration in buffer can also be used for prediction of the in vivo plasma exposure-based $CL_{int,bile}$ with correction for protein binding, pairs of transporters involved in uptake and biliary excretion, and a large scaling factor (up to 300-fold) to bridge the in vitro to in vivo gap (Fukuda et al., 2008; Nakakariya et al., 2012; Zou et al., 2013).

Zou et al. (2013) suggest several reasons why a larger scaling factor is required for compounds with low $CL_{int,bile,app}$. For instance, a measurement error for compounds with low biliary excretion and/or high protein binding leads to poor prediction. In addition, to achieve good response on measurement for compounds with low uptake to hepatocytes, those are often incubated at high concentration in protein-free buffer. The high concentration in the buffer may partially saturate uptake and/or efflux

transporters in SCHs, leading to underestimation. They also suggest that addition of serum protein or plasma/serum itself to the buffer can reduce the prediction error because serum protein serves just as a drug solubilizer and does not remarkably change hepatic uptake of lipophilic compounds with high protein binding.

In addition to excretion across the canalicular membranes, SCHs allow investigation of basolateral uptake and efflux transport processes. The major drug-associated uptake transporters in human liver are OATPs, sodium taurocholate cotransporting polypeptide (NTCP), and organic cation transporter 1 (OCT1) (Fig. 2), and all of these transporters function in human SCHs. The activities of uptake transporters in SCHs depend on the culture condition and species, and are usually similar to or less than those in other forms of hepatocytes (Hoffmaster et al., 2005; Bi et al., 2006; De Bruyn et al., 2011; Jacobsen et al., 2011; Kotani et al., 2011; Tchapanian et al., 2011). Comparisons of uptake transporter activities between SCHs and hepatocytes in suspension are summarized in Table 1.

A recent meta-analysis showed that the levels of OATP1B1 and OATP1B3 do not differ significantly between human liver tissue and human SCHs, but that there is a difference for OATP2B1 (Badée et al., 2015). In contrast, protein levels of major rat Oatps, Oatp1a1, Oatp1a4, and Oatp1b2, are significantly lower in SCHs than those in liver tissues (Ishida et al., 2018). MRP3 and MRP4 expressed on the basolateral membrane of hepatocytes are involved in the efflux of numerous endogenous and exogenous compounds.

A vesicle-based system is suitable to investigate involvement and interaction of efflux transporters; however, it is still unclear if vesicle-based data can be used to predict sinusoidal efflux and biliary excretion clearance, and the magnitude of the interaction (Brouwer et al., 2013). In contrast, SCHs retain both basolateral and canalicular efflux transporter networks, and the functional involvement of MRP3 and/or MRP4 has been reported in efflux of rosuvastatin, enalaprilat, and mycophenolic acid phenyl-glucuronide (MPAG) in SCHs (Pfeifer et al., 2013a; Ferslew et al., 2014; Matsunaga et al., 2015). Enalaprilat and MPAG are formed in the liver and undergo translocation into the systemic circulation. These findings highlight that comprehensive analyses of efflux processes is essential to predict drug disposition in vivo, especially for metabolites because intracellular formation of a metabolite is the first step, followed by excretion into the bloodstream and/or bile (enzyme–efflux transporter interplay).

In addition, a metabolite is usually more hydrophilic and less membrane permeable than a parent compound (Pang et al., 1984; de Lannoy and Pang, 1987) and involvement of non-P450 enzymes, especially UGTs, has increased because of the effort to escape P450-mediated metabolism and improve metabolic stability during drug discovery and development (Argikar et al., 2016). Therefore, efflux transporters may have a major role in transport of a metabolite from the liver to the blood and/or bile. Therefore, SCHs are a suitable model to investigate the hepatic disposition of both a parent drug (mainly uptake transporter–enzyme interplay) and an intracellularly formed metabolite (mainly enzyme–efflux transporter interplay). On the other hand, in TR^- rats, genetically *Mrp2*-deficient rats, glucuronidation activities are higher than those in wild-type Wistar rats (Westley et al., 2008; Yang and Brouwer, 2014), suggesting UGT activities in TR^- rat SCHs are higher than those in wild-type Wistar rat SCHs. Accordingly, it should be noted that functional change of canalicular transporters may lead to functional change of drug-metabolizing enzymes.

Prediction of Species Differences in Hepatic Disposition of Metabolites

Differences in biliary excretion of drugs have been reported across species (Grime and Paine, 2013). Molecular weight is one of determinant

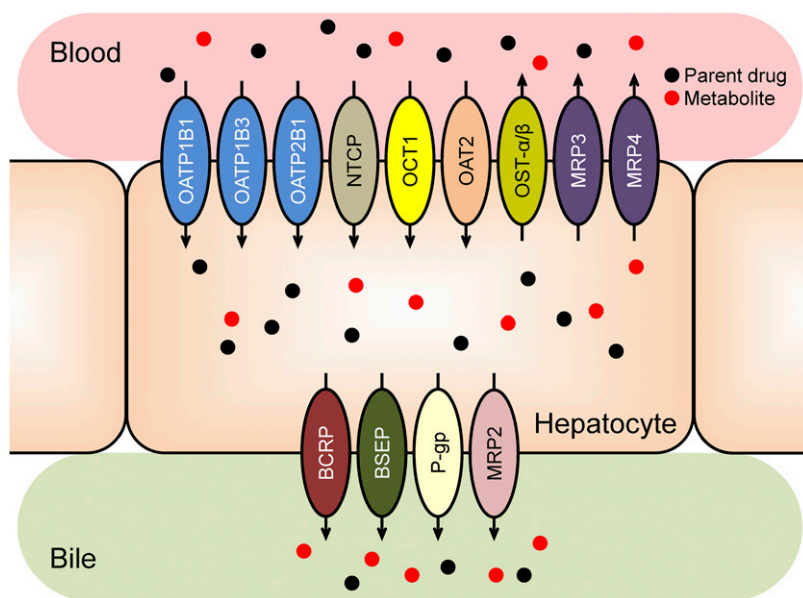


Fig. 2. Major drug-associated transporters in human liver. OATPs (OATP1B1, OATP1B3, and OATP2B1), NTCP, organic cation transporter 1 (OCT1), and organic anion transporter OAT2 are major uptake transporters in human liver. MRP3, MRP4, and heteromer organic solute transporters OST- α/β are expressed on the same basolateral membrane and are involved in efflux from hepatocytes to the bloodstream. BCRP, BSEP, P-gp, and MRP2 are major canalicular efflux transporters involved in biliary excretion (Hillgren et al., 2013).

of substrates for canalicular efflux transporters BCRP and MRP2, with different molecular weight thresholds in rats and humans (Kato et al., 2008; Choi et al., 2009; Yang et al., 2009). For anionic compounds, a molecular weight higher than 400 and 475 g/mol is assumed the cutoff value in rats and humans, respectively, whereas no threshold molecular weight is identified for cationic and neutral compounds either in rats or humans.

In addition, recent quantitative structure–activity relationship analyses have shown some features of canalicular transporters for substrate recognition. For instance, P-gp substrates are bulky, highly branched, and good electron acceptors. BCRP substrates contain a large, positively charged surface and have aromatic rings. MRP2 substrates are flexible in addition to having a large polar and hydrophilic surface area. P-gp and BCRP are more associated with explicit aromaticity-related features, whereas MRP2 is predominately associated with hydrophilicity-related properties (Aniceto et al., 2016).

Because a metabolite, especially a conjugated metabolite, has a higher molecular mass and hydrophilicity than the parent drug, the metabolite is more likely to be a substrate of a canalicular efflux transporter, as shown in Table 2. Some reports have indicated that basolateral and canalicular efflux in SCHs are correlated with *in vivo* excretion of metabolites into urine or bile. For instance, paroxetine is mainly metabolized in the liver and excreted in the form of metabolites. M1-glucuronide, a major metabolite of paroxetine, is mainly excreted into bile after oral administration in rats. In humans, in contrast, M1-sulfate and M1-

glucuronide are major metabolites and are mainly excreted in urine (Haddock et al., 1989; Kaye et al., 1989). In rat SCHs, M1-glucuronide was found to be the main metabolite, with minimal levels of other metabolites, including M1-sulfate. In contrast, similar levels of M1-glucuronide and M1-sulfate were observed in human SCHs. The BEIs of intracellularly formed M1-glucuronide were ~50.9% in rats and ~15.6% in humans, respectively (Matsunaga et al., 2013), which is consistent with the main elimination routes of M1-glucuronide in rats and humans.

Similar data have been reported for mycophenolic acid (MPA), which is mainly metabolized to MPAG in the liver (Shipkova et al., 2001). In rats, MPAG is excreted in bile and urine, whereas MPAG is mainly excreted in urine in humans (Bullingham et al., 1998; Gao et al., 2011). The contributions of canalicular efflux of intracellularly formed MPAG to net (basolateral and canalicular) efflux were estimated to be 37% in rat SCHs and 20% in human SCHs, suggesting species differences in the direction of basolateral and canalicular efflux of MPAG (Tetsuka et al., 2014b).

These independent reports using SCH systems suggest that species differences in urinary and fecal/biliary excretion of M1-glucuronide and MPAG depend on biliary excretion availability because these metabolites are substrates of MRP2/Mrp2. For troglitazone, in contrast, there are no significant species differences in the metabolism of the parent drug and the urinary/biliary excretion balance of the main metabolite (troglitazone sulfate) between rat and human SCHs, and these is also

TABLE 1
Activities of uptake transporters in SCHs and hepatocytes in suspension

Substrate	Accumulation (pmol/min/mg protein or 10^6 cells) or Uptake Clearance ($\mu\text{L}/\text{min}/\text{mg}$ protein)		References
	SCHs	Suspended Hepatocytes	
CCK-8 (human)	1.06 $\mu\text{L}/\text{min}/\text{mg}$ protein	2.44 $\mu\text{L}/\text{min}/\text{mg}$ protein	Kotani et al. (2011)
Digoxin (human)	3.8 \pm 1.7 pmol/min per 10^6 cells	5.2 \pm 2.6 pmol/min per 10^6 cells	De Bruyn et al. (2011)
Estrone-3-sulfate (human)	30.3 \pm 13.0 pmol/min per 10^6 cells	99.1 \pm 67 pmol/min per 10^6 cells	De Bruyn et al. (2011)
1-Methyl-4-phenylpyridinium (MPP+) (rat)	~17 pmol/min/mg protein	91 pmol/min/mg protein	Jacobsen et al. (2011)
Pravastatin (human)	2.95 $\mu\text{L}/\text{min}/\text{mg}$ protein	2.17 $\mu\text{L}/\text{min}/\text{mg}$ protein	Kotani et al. (2011)
Rosuvastatin (human)	9.48 $\mu\text{L}/\text{min}/\text{mg}$ protein	9.50 $\mu\text{L}/\text{min}/\text{mg}$ protein	Kotani et al. (2011)
TCA (human)	20.0 \pm 7.9 pmol/min per 10^6 cells	27.5 \pm 15 pmol/min per 10^6 cells	De Bruyn et al. (2011)
TCA (rat)	40.8 \pm 5.7 pmol/min/mg protein at 10 μM	52.8 \pm 10.2 pmol/min/mg protein at 1 μM	Kemp et al. (2005)

TABLE 2
Hepatic transporters involved in parent drug and metabolite pairs

Drugs and Metabolites	Basolateral Uptake Transporters	Basolateral Efflux Transporters	Canalicular Efflux Transporters	References
Bilirubin	OATP1B1, OATP1B3	NA	NA	Lengyel et al. (2005);
Bilirubin mono/diglucuronide	OATP1B1	Mrp1, MRP3	BCRP, MRP2/Mrp2	van de Steeg et al. (2012); Chang et al. (2013)
Diclofenac	Not substrate of either OATP1B1, OATP1B3, or OATP2B1	Not substrate of MRP3	Not substrate of either BCRP or MRP2	Zhang et al. (2016)
Diclofenac acyl glucuronide	OATP1B1, OATP2B1	MRP3	BCRP, MRP2	
Enalapril	OATP1B1, OATP1B3	Not substrate of either MRP3 or MRP4	MRP2/Mrp2	Liu et al. (2006); Ferslew et al. (2014)
Enalaprilat	NA	MRP4	MRP2/Mrp2	
Ezetimibe	Not substrate of either OATP1B1, OATP1B3 or OATP2B1	NA	Not substrate of MRP2	Oswald et al. (2008); Fahrmayr et al. (2012)
Ezetimibe glucuronide	OATP1B1, OATP2B1	NA	MRP2	
Irinotecan	Not substrate of OATP1B1	NA	P-gp, MRP2	Nozawa et al. (2005);
SN-38	OATP1B1	NA	BCRP, P-gp, MRP2	Di Martino et al. (2011)
SN-38 glucuronide	Not substrate of OATP1B1	NA	MRP2	
Morphine	OCT1	Not substrate of MRP3	P-gp	Tzvetkov et al. (2013);
Morphine 6-glucuronide	NA	MRP3	P-gp, MRP2	Venkatasubramanian et al. (2014)
Paroxetine	NA	NA	NA	Matsunaga et al. (2013)
M1-glucuronide	NA	NA	MRP2/Mrp2	
M1-sulfate	NA	NA	Not substrate of either MRP2/Mrp2, BCRP/Bcrp, or BSEP/Bsep	
Mycophenolate mofetil	NA	NA	NA	Picard et al. (2010);
Mycophenolic acid (MPA)	Not substrate of OATPs	NA	NA	Matsunaga et al. (2014);
MPA phenyl-glucuronide (MPAG)	OATP1B1, OATP1B3	MRP3, MRP4	MRP2/Mrp2	Tetsuka et al. (2014b)
Troglitazone	NA	NA	NA	Enokizono et al. (2007);
Troglitazone sulfate	OATP1B1	Mrp3, Mrp4	BCRP, MRP2	Lee et al. (2010)

NA, not applicable; SN-38, 7-ethyl-10-hydroxycamptothecin.

in good agreement with the *in vivo* disposition of troglitazone sulfate in both species (Lee et al., 2010). These findings show that SCHs can reproduce the *in vivo* hepatic disposition of a parent drug and metabolite and predict species differences.

OATP1B1 and OATP1B3 are involved in bilirubin and its glucuronide uptake to the liver. In addition, UGT1A1 catalyzes bilirubin conjugation to monoglucuronide (BMG) and diglucuronide (BDG), and subsequent biliary excretion is mediated by MRP2. Drug-induced hyperbilirubinemia is thought to be caused by inhibition of either UGT1A1 or these transporters (Chang et al., 2013; Keppler, 2014). The sum of BEIs of the formed bilirubin glucuronides (BMG and BDG) was 80.6% in rat SCHs and 62.5% in human SCHs, suggesting that bilirubin glucuronides are preferably excreted into the canalicular networks in both rats and humans. In rat SCHs, formations of BMG and BDG were comparable, whereas the excreted amount of BDG into bile canaliculi was higher than that of BMG in rat SCHs (Lengyel et al., 2005). The kinetic parameters of rat Mrp2 were $0.8 \pm 0.2 \mu\text{mol/l}$ of K_m and $213 \pm 52 \mu\text{l/min}$ per milligram protein of CL_{int} for BMG and $0.5 \pm 0.1 \mu\text{mol/l}$ of K_m and $520 \pm 24 \mu\text{l/min}$ per milligram protein of CL_{int} for BDG, respectively (Kamisako et al., 1999), suggesting that BDG is a better-transported substrate of rat Mrp2, which is consistent with *in vivo* observations. In addition, the BDG/BMG ratio in the medium (0.55) and bile canaliculi (1.48) in rat SCHs was comparable to those in serum (0.6) and bile (1.5) of rats (Mesa et al., 1997; Lengyel et al., 2005). These findings suggest that SCHs enable evaluation of basolateral and canalicular membrane transport of bilirubin glucuronides.

The BDG/BMG ratio in bile and serum changes in different types of hyperbilirubinemia. For instance, in Gilbert and Crigler-Najjar syndromes, which are caused by genetic polymorphisms of UGT1A1 and exhibit decreased or deficient glucuronidation activities, there is a shift toward BMG, consistent with a decrease of the BDG/BMG ratio in bile and serum in Gunn rats, an animal model of Crigler-Najjar syndrome (Van Steenberg and Fevery, 1990; Clarke et al., 1997). In contrast,

bile duct-ligated and Mrp2-deficient rats, a model of Dubin-Johnson syndrome, have an increased BDG/BMG ratio in bile and serum, corresponding to clinical findings in patients with extrahepatic cholestasis (Jansen et al., 1985; Sieg et al., 1986; Mesa et al., 1997). These results show that the mechanism of drug-induced hyperbilirubinemia could be explored by measuring the BDG/BMG ratio in SCHs. To establish the usefulness of the BMG/BDG ratio change in SCHs for investigating drug-induced hyperbilirubinemia, further studies are essential using a number of drugs that cause hyperbilirubinemia.

Probe Selection to Evaluate Drug-Drug Interactions via Canalicular Efflux Transporters

SCHs can form a bile canalicular network, and this allows investigation of intact cell-based interactions with canalicular transporters. Among the canalicular transporters expressed in hepatocytes, BCRP, BSEP, P-gp, and MRP2 are important efflux transporters that are involved in drug-drug interactions (DDIs). MRP2 substrates are mostly conjugated metabolites, including MPAG and BMG/BDG, and MRP2 inhibition indirectly leads to a DDI via the decrease of enterohepatic circulation of metabolites such as MPAG or results in conjugated hyperbilirubinemia. In addition, inhibition of BSEP is considered to cause liver injury (Hillgren et al., 2013).

Several fluorescent or radiolabeled probes have been used to determine the interaction with canalicular efflux transporters in SCHs, but these probes are often substrates of both basolateral uptake and canalicular efflux transporters. For instance, estrone 3-sulfate, taurocholate (TCA), rhodamine 123, and estradiol 17 β -D-glucuronide (E17G) are generally used as model substrates for BCRP, BSEP, P-gp, and MRP2, respectively; however, they are also substrates of OATPs and/or NTCP (Annaert and Brouwer, 2005; McRae et al., 2006; Fukuda et al., 2008; Brouwer et al., 2013; Pedersen et al., 2013). In addition, some probes, such as E17G and TCA, are substrates of basolateral efflux transporter

MRP3 and/or MRP4 (Hirohashi et al., 1999; Hirohashi et al., 2000; Akita et al., 2002; Rius et al., 2006).

BEI is frequently used as a marker for inhibition of canalicular transport in SCHs and is determined from intracellular and bile canaliculi-excreted accumulations of substrate, based on eq. 2. Because intracellular substrate accumulation is affected by basolateral uptake and efflux as well as by metabolism, the assessment of canalicular transporter-mediated interactions based on BEI is complicated. Caution should be taken as to whether the perpetrator exhibits inhibitory potential for multiple transporters and/or drug metabolizing enzymes in hepatocytes.

The fluorescent probe 5(6)-carboxy-2',7'-dichlorofluorescein (CDF) is a substrate of MRP2 and is used as a marker of MRP2 function; however, CDF is also a substrate of basolateral uptake and efflux transporters OATPs and MRP3 (Zamek-Gliszczynski et al., 2003; Ellis et al., 2014). In contrast, 5(6)-carboxy-2',7'-dichlorofluorescein diacetate (CDFDA) is a nonfluorescent ester form of CDF with uptake into hepatocytes that is not saturable, temperature dependent, or impaired by transporter inhibitors (Zamek-Gliszczynski et al., 2003), suggesting that CDFDA crosses the membrane mainly by passive diffusion. Once CDFDA is in the cells, it is rapidly hydrolyzed by intracellular esterases to the fluorescent probe CDF, which is subsequently actively transported out to bile canaliculi by MRP2 in SCHs.

This enzyme–efflux transporter interplay of CDFDA/CDF has been applied in the SCH system as a quantitative time-lapse imaging (QTLI) method to investigate MRP2-mediated interaction by time-dependent detection of fluorescent dye in bile canaliculi of SCHs, regardless of whether the perpetrator has an inhibitory potential for basolateral uptake transporters (Nakanishi et al., 2011, 2012).

Most of the MRP2 substrates are not parent drugs but conjugated metabolites, and the inhibition causes the decrease of the enterohepatic circulation, leading to an indirect DDI. MRP2 inhibition also results in conjugated hyperbilirubinemia. Therefore, investigation of MRP2 inhibition is considered clinically relevant. In addition, as well as other transporters such as OATPs (Noé et al., 2007; Tamai and Nakanishi, 2013; Hoshino et al., 2016), MRP2 is considered to have multiple binding sites (Gilibili et al., 2017). A typical MRP2 substrate E17G follows the sigmoidal kinetics in MRP2-mediated uptake to membrane vesicles with high and low affinities, whereas the uptake of CDF and an endogenous substrate coproporphyrin-I (CP-I) exhibits the simple Michaelis-Menten kinetics, suggesting CDF and CP-I are the more preferred substrates for investigation of MRP2 inhibition. Although CP-I is endogenous and may be used as a biomarker of MRP2 function in vivo, it is also a substrate of OATPs (Shen et al., 2016). Accordingly, QTLI using CDFDA allows direct measurement of fluorescent dye

accumulation in bile canaliculi over time without calcium/magnesium depletion, giving a time- and cost-effective in vitro system for screening for MRP2 inhibition at an early stage of drug development.

QTLI also enables prediction of apparent inhibition of MRP2 by metabolites formed in the hepatocytes, even when the metabolites are not identified. A similar QTLI method has been used to investigate BSEP function using a fluorescent bile acid (BA) derivative *N*-(24-[7-(4-*N,N*-dimethylaminosulfonyl-2,1,3-benzoxadiazole)]amino-3 α ,7 α ,12 α -trihydroxy-27-nor-5 β -cholestan-26-oyl)-2'-aminoethanesulfonate (tauro-nor-THCA-24-DBD). However, the uptake of tauro-nor-THCA-24-DBD is mediated by NTCP and OATPs and affected by their inhibitors (De Bruyn et al., 2014). Rhodamine 123 is a fluorescent substrate used for assessment of P-gp function that can be applied for QTLI analysis; however, it is also an Oatp substrate in rats (Annaert and Brouwer, 2005). In many cases, interactions with basolateral uptake transporters must be taken into account in hepatobiliary transport in SCHs because the probe is often a substrate of both basolateral uptake and canalicular efflux transporters. Therefore, enzyme–efflux transporter interplay in SCHs may allow separation of involvement of basolateral uptake transporters from hepatobiliary transport using more selective probes such as CDFDA/CDF.

More Clinically Relevant Prediction of DDIs by Enzyme–Transporter Interplay

Enzyme–efflux transporter interplay in SCHs is sometimes useful for prediction of more clinically relevant DDIs. Candesartan cilexetil (CCX) is rapidly hydrolyzed to the active form candesartan (CAN) by esterases in the liver. CCX is a more potent inhibitor of human BSEP than CAN in vesicular transport assays using membrane vesicles prepared from BSEP-expressing cells (IC_{50} values of CCX and CAN for human BSEP are 6.2 and 70.5 μ M, respectively), whereas exposing CCX to human SCHs did not affect the hepatobiliary transport of TCA (66.5% in control versus 62.7% in CCX as BEI) (Fukuda et al., 2014). Diisopropyl fluorophosphate, an irreversible cholinesterase inhibitor, significantly inhibited the metabolism of CCX to CAN in human SCHs and resulted in a significant decrease of BEI for TCA due to an increase of intracellular CCX concentration and subsequent inhibition of human BSEP activity (Fukuda et al., 2014). These findings are consistent with the low risk of CCX for liver dysfunction or jaundice in clinical use (Fig. 3A).

Another example is a situation in which the metabolite causes a DDI, rather than the parent. Estradiol (E_2) itself is not a substrate of MRP2, but its metabolite E17G is a substrate. E_2 does not affect rat MRP2-mediated uptake of CDF up to 300 μ M in vesicular transport experiments,

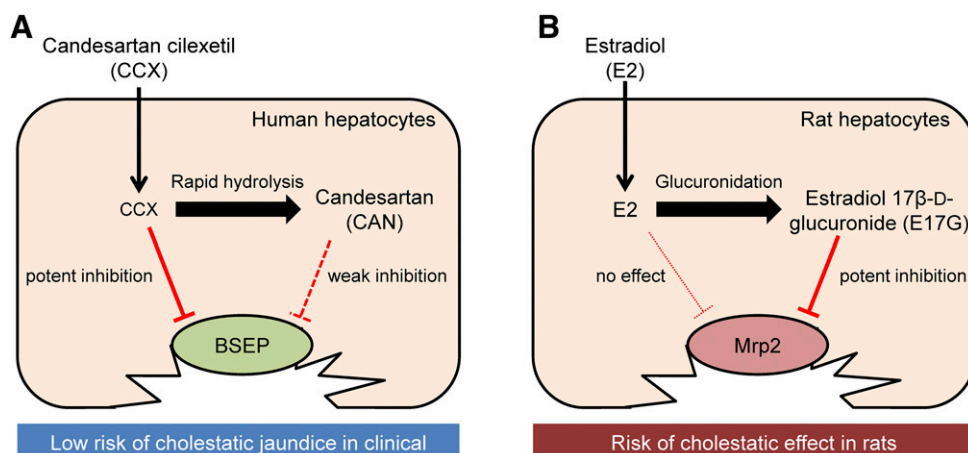


Fig. 3. SCHs allows clinically and in vivo relevant prediction of DDIs caused by both parent drug and metabolite hepatic exposure. (A) Candesartan cilexetil (CCX) is a potent human BSEP inhibitor, but it is rapidly hydrolyzed in hepatocytes to candesartan (CAN), which is a weak inhibitor of BSEP, consistent with the low risks of cholestatic jaundice in clinical use of CCX (Fukuda et al., 2014). (B) Estradiol (E_2) has no potency for rat MRP2, but its metabolite, estradiol 17 β -D-glucuronide (E17G), is a potent MRP2 inhibitor, leading to acute cholestasis in rats (Meyers et al., 1980, 1981; Nakanishi et al., 2012).

TABLE 3

Established mathematical models of parent and metabolites obtained from SCH-based data

Drugs and Metabolites	Remarks	References
Terfenadine	~15% as BEI, $k_{\text{efflux}} \gg k_{\text{bile}}$ in rat SCHs	Turncliff et al. (2006)
Fexofenadine	~15% as BEI, $k_{\text{efflux}} > k_{\text{bile}}$ in rat SCHs	
Troglitazone	Minimum biliary excretion in rat SCHs	Lee et al. (2010)
Troglitazone glucuronide	~50% as BEI, $k_{\text{efflux}} > k_{\text{bile}}$ in rat SCHs	
Troglitazone sulfate	~20% as BEI, $k_{\text{efflux}} < k_{\text{bile}}$ in rat SCHs	
Pafuramidine	Rapidly metabolized in rat and human SCHs	Yan et al. (2011, 2012)
Furamidine (active form of pafuramidine)	$k_{\text{efflux}} > k_{\text{bile}}$ in rat and human SCHs	
CPD-0868	Rapidly metabolized in rat SCHs	Yan et al. (2011)
CPD-0801 (active form of CPD-0868)	$k_{\text{efflux}} > k_{\text{bile}}$ in rat SCHs	
Mycophenolic acid (MPA)	Rapidly metabolized in human SCHs	Matsunaga et al. (2014)
MPA phenyl-glucuronide (MPAG)	~40% as BEI, $k_{\text{efflux}} > k_{\text{bile}}$ in human SCHs	
Bosentan	Minimum biliary excretion in human SCHs	Matsunaga et al. (2016)
Ro 48-5033	$k_{\text{efflux}} = k_{\text{bile}}$ in human SCHs	
Ro 47-8634	$k_{\text{efflux}} = k_{\text{bile}}$ in human SCHs	
Ro 64-1056	$k_{\text{efflux}} = k_{\text{bile}}$ in human SCHs	

BEI, biliary excretion index; CPD-0801, 2,5-bis (5-amidino)-2-pyridyl furan; CPD-0868, 2,5-bis [5-(*N*-methoxyamidino)-2-pyridyl] furan; k_{bile} , biliary excretion rate from cells to bile canaliculi; k_{efflux} , efflux rate from cells to buffer; OCT1, organic cation transporter 1.

whereas E17G showed preloading time- and concentration-dependent inhibition of Mrp2 function in rat SCHs using QTLI with CDFDA/CDF (Nakanishi et al., 2012). Because the preloading time- and concentration-dependent formation of E17G was observed after adding E₂ to rat SCHs, and E17G directly inhibited CDF uptake in Mrp2-expressing vesicles in a concentration-dependent manner (Nakanishi et al., 2012), the effect of E₂ on Mrp2 function in rat SCHs is apparently indirect, via formation of E17G (Fig. 3B). This corresponds to in vivo acute cholestasis in rats after administration of E₂ (Meyers et al., 1980, 1981). Therefore, SCHs can provide clinically and in vivo relevant DDI predictions based on hepatic exposures of both a parent drug and intracellularly formed metabolites.

Utility of Mechanistic Modeling of Hepatic Disposition of Parent Drugs and Metabolites

The utility of modeling and simulation in drug development has been widely recognized and illustrated (Huang et al., 2013; Milligan et al., 2013). Some multicompartment models are useful for understanding the dynamic disposition of the parent drug and metabolites mechanistically in hepatocytes. Cryopreserved hepatocytes are more convenient and flexible to study drug transport compared with freshly isolated hepatocytes. There is debate about changes of uptake transporter activities in hepatocytes during cryopreservation, with similar or fewer uptake activities in suspended hepatocytes after thawing (Houle et al., 2003; Badolo et al., 2011; Brouwer et al., 2013; Lundquist et al., 2014). In addition, There is some debate on efflux transporter function in suspended or monolayer hepatocytes, but in general these transporters are assumed to be internalized after isolation or short-term culturing and their activities can be negligible, especially in short-term incubation (Hewitt et al., 2007; Jørgensen et al., 2007; Bow et al., 2008).

In contrast, SCHs are expected to retain an entire array of transporter and drug metabolizing-enzyme activities in the liver, and all four processes of hepatic disposition can be simultaneously determined in SCHs. Accordingly, modeling SCH-based data enables simultaneous estimation of in vitro transporter- and enzyme-mediated parameters, in addition to passive diffusion and intracellular binding.

Three compartments (buffer, cell, and bile canaliculi) are generally used for mathematical modeling in SCHs, shown in Supplemental Figure 1. Rich input data obtained by monitoring parent drug and metabolites over time are necessary to estimate the parameters precisely. A flux from bile canaliculi to buffer is technically present in SCHs, and

this has to be taken into account in the model (Matsunaga et al., 2014). For estimating basolateral efflux, at least two methods (one- or two-step) have been proposed (Turncliff et al., 2006; Lee et al., 2010; Pfeifer et al., 2013a; Matsunaga et al., 2014, 2016; Guo et al., 2016). Establishing an in vitro mechanistic model leads to further analyses, such as sensitivity analysis, DDI prediction, and identification of a novel metabolic pathway (Lee et al., 2010; Matsunaga et al., 2014, 2015, 2016; Guo et al., 2016). In vitro parameter estimates from SCH-based data can also be extrapolated to the whole liver level and subsequently incorporated into physiologically based pharmacokinetics models (Yan et al., 2011; Yan et al., 2012; Pfeifer et al., 2013b). Mathematical models of pairs of parent drug and metabolite in SCHs are summarized in Table 3.

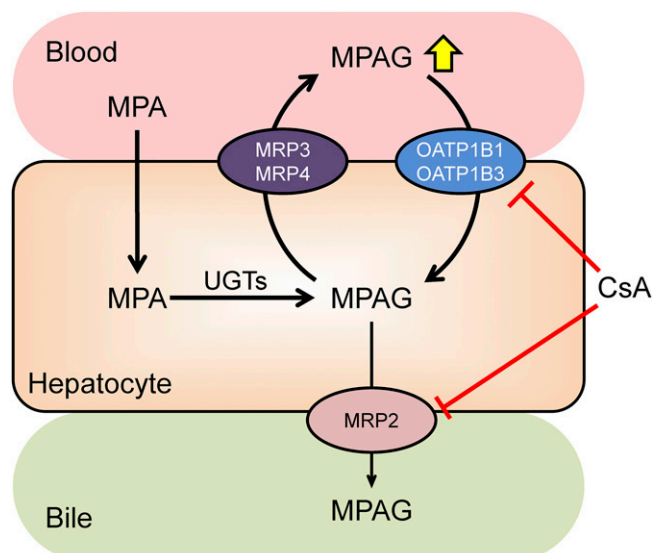


Fig. 4. Hepatic disposition of MPA and MPAG and DDI with CsA. MPA is predominantly metabolized to MPAG in human liver. The formed MPAG is transported to the bloodstream by basolateral efflux transporters MRP3 and MRP4 and to the bile by the canalicular efflux transporter MRP2 (Matsunaga et al., 2014). Concomitant drug CsA inhibits OATP-mediated uptake and MRP2-mediated canalicular efflux of MPAG at clinically relevant concentrations, and subsequent systemic exposure of MPAG is increased (Naito et al., 2009; Yau et al., 2009).

Modeling of Hepatic Disposition of MPA and MPAG and Interactions with Cyclosporine A in Human SCHs

MPA is widely used as an immunosuppressive agent, especially after renal transplantation, and is predominantly metabolized to MPAG (Shipkova et al., 2001). MPAG is considered to undergo biliary excretion and to contribute to the continuous systemic exposure of MPA via the enterohepatic circulation. However, the systemic exposure of MPAG is much greater than that of MPA and the administered MPA is eventually excreted in urine at ~90% of the dose as MPAG (Bullingham et al., 1998). In human SCHs, MPA was primarily converted to MPAG, and the formed MPAG was a preferred substrate of MRP2 with ~50% BEI. In contrast, mechanistic modeling of MPA and MPAG disposition in human SCHs revealed that the basolateral efflux rate of formed MPAG was 2.4 times faster than the canalicular efflux rate, corresponding to the *in vivo* observation that MPAG is excreted into bile but the systemic exposure is high. MPAG is a substrate of basolateral efflux transporters MRP3 and MRP4, and the sensitivity analysis of the model suggested a significant impact of functional change of basolateral efflux activities on MPAG disposition (Matsunaga et al., 2014).

MPA is usually used in combination with a calcineurin inhibitor, such as cyclosporin A (CsA) or tacrolimus, in immunosuppressive therapy (Staatz and Tett, 2007). CsA is a well-known inhibitor of several drug metabolizing-enzymes and transporters in clinical use (Zhang et al., 2008; Liu et al., 2011). Indeed, concurrent administration of CsA causes decreased exposure of MPA in patients by inhibition of MRP2-mediated biliary excretion and subsequent reduction of enterohepatic circulation of MPAG (Smak Gregoor et al., 1999; Hesselink et al., 2005; Tetsuka et al., 2014b). In human SCHs, CsA decreased the BEI of MPAG and increased intracellular MPAG accumulation in a concentration-dependent manner, whereas CsA did not affect MPA disposition or formation of MPAG (Matsunaga et al., 2015).

The established *in vitro* hepatic disposition model of MPA and MPAG in human SCHs was applied to investigate the effects of CsA on MPAG disposition, and revealed that CsA had different inhibitory potencies for basolateral and canalicular efflux transporter-mediated MPAG disposition of ~50% and ~80%, respectively, as maximum inhibition. In addition, model-based analysis suggested that at clinically relevant concentrations of CsA, the extracellular medium concentration of MPAG was increased by inhibition of OATP-mediated uptake and

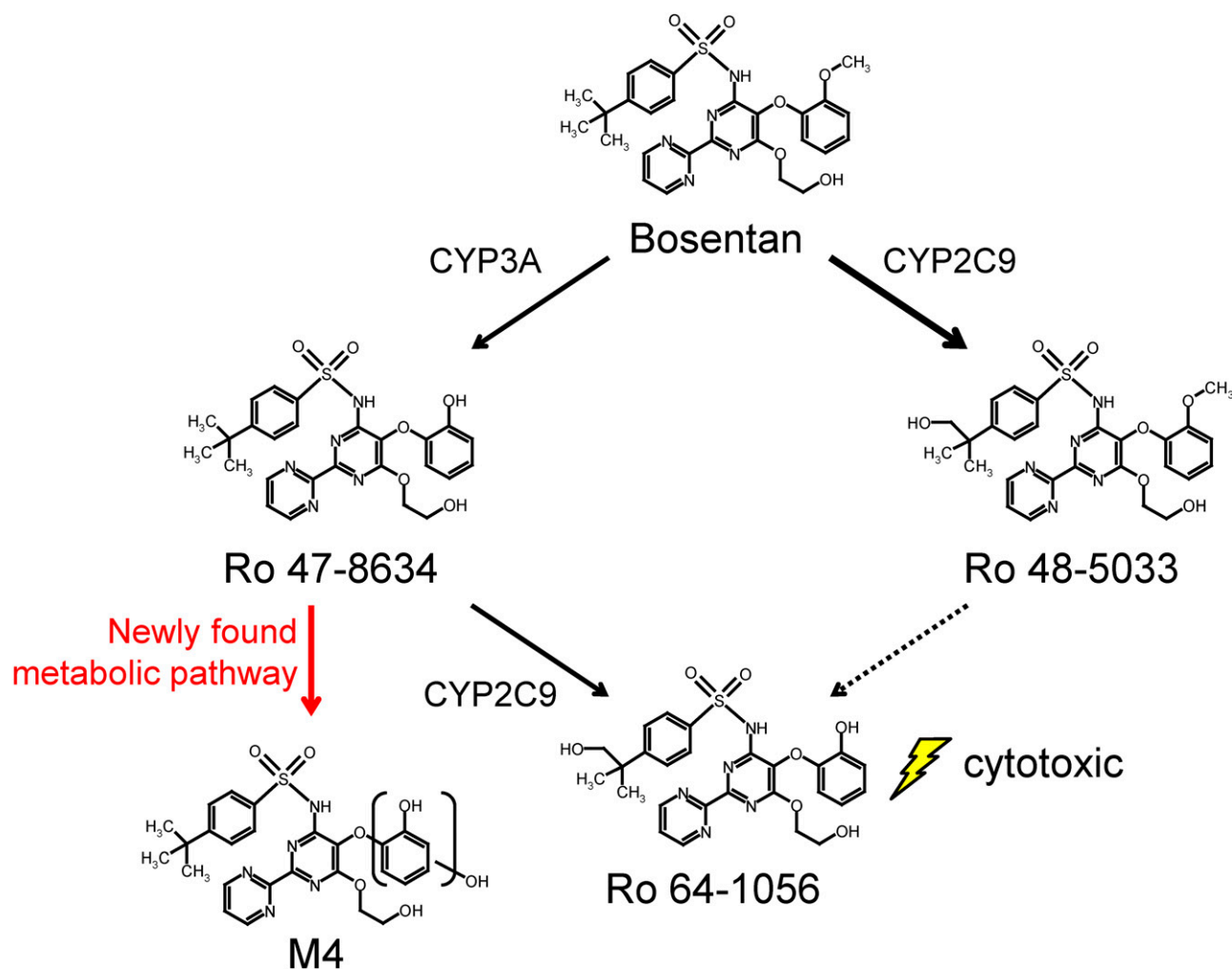


Fig. 5. Hepatic disposition of bosentan and its metabolites in human liver. Bosentan is metabolized by CYP2C9 and CYP3A to Ro 48-5033 and Ro 47-8634. Ro 47-8634 is further metabolized to Ro 64-1056 and M4, a novel metabolite found by a modeling approach (Matsunaga et al., 2016). Ro 64-1056 exhibits concentration-dependent cytotoxicity in human hepatocytes, and the metabolic pathway from Ro 47-8634 to M4 is considered to be an alternative route that avoids Ro 64-1056-induced hepatotoxicity.

MRP2-mediated biliary excretion of MPAG (Matsunaga et al., 2015) (Fig. 4). This result is consistent with clinical observations (Naito et al., 2009; Yau et al., 2009). Thus, the MPA and MPAG pair is a good example with which to understand the utility of mathematical modeling of SCH-based data.

Identification of Novel Metabolic Pathways and Improved Understanding of Hepatotoxicity

A quantitative mechanistic analysis can provide previously unknown information. Bosentan, an oral nonselective endothelin receptor antagonist, is metabolized in human liver to Ro 48-5033 (hydroxylated form [4-(2-hydroxy-1,1-dimethyl-ethyl)-N-[6-(2-hydroxy-ethoxy)-5-(2-methoxyphenoxy)-[2,2#1;]bipyrimidinyl-4-yl]-benzenesulfonamide]) or Ro 47-8634 (O-demethylated form [4-tert-butyl-N-[6-(2-hydroxyethoxy)-5-(2-hydroxyphenoxy)-2-(pyrimidin-2-yl)pyrimidin-4-yl]benzene-1-sulfonamide]) and is subsequently metabolized to Ro 64-1056 (combination of hydroxylation and O-demethylation [4-(1-hydroxy-2-methylpropan-2-yl)-N-[5-(2-hydroxyphenoxy)-2-pyrimidin-2-yl-6-(1,1,2,2-tetradeuterio-2-hydroxyethoxy)pyrimidin-4-yl]benzenesulfonamide]) (Matsunaga et al., 2016).

Bosentan is a hepatotoxic drug, but its mechanism is not fully understood. In human SCHs, the total recovered amounts of bosentan and its three metabolites after incubation was 88.3%–111.9% of the dose added, but a mathematical model of known metabolic pathways failed to predict the disposition of the drug and metabolites in human SCHs. An unknown metabolite peak was found in bioanalyses of SCH samples, which was determined to be a hydroxylated form of Ro 47-8634 and named M4 (Matsunaga et al., 2016). A modified model including the new M4 pathway successfully predicted the disposition of bosentan and its metabolites in human SCHs. The pathway from Ro 47-8634 to M4 is an alternative route avoiding Ro 64-1056–induced hepatotoxicity because Ro 64-1056 decreased cell viability in a concentration-dependent manner and the cytotoxicity was ameliorated by inhibition of metabolism of Ro 47-8634 to Ro 64-1056 (Fig. 5).

Drug-induced liver injury is triggered by various mechanisms, including reactive metabolite formation and mitochondrial dysfunction, and BSEP inhibition is a risk factor for hepatotoxicity due to cholestasis. As SCHs can form a canalicular network, SCH-based models have been proposed to examine drug-induced cholestasis in combination with a BA mixture (Ogimura et al., 2011; Chatterjee et al., 2014a; Oorts et al., 2016). In some cases, metabolites show similar or more potent inhibition of BSEP function, e.g., Ro 47-8634 (bosentan) and DM-4103 (tolvaptan) (Fattinger et al., 2001; Slizgi et al., 2016); therefore, in vitro modeling of the parent drug and metabolite in SCHs is informative for understanding the mechanism of the cholestatic effect by metabolic activation.

It is also of note that BA composition differs between rats and humans and each BA exhibits different properties of hydrophobicity, toxicity, and interplay with multiple transporters and enzymes (Alvaro et al., 1986; Tagliacozzi et al., 2003; Chatterjee et al., 2014b; Rodrigues et al., 2014). In addition, unconjugated BA is conjugated with glycine or taurine in SCHs during incubation (Marion et al., 2011), and bile acid CoA:amino acid N-acyltransferase catalyzes this reaction. As some genetic polymorphisms altering catalytic properties are found in coding regions of the enzyme (Tougou et al., 2007), a functional change of this enzyme may also influence BA-related hepatotoxicity. Accordingly, in addition to the analysis of disposition of a perpetrating drug and its metabolite, mathematical modeling of BA composition in SCHs should be useful to understand BA-triggered hepatotoxicity mechanistically.

Conclusion

To date, transporters and drug-metabolizing enzymes have been identified and characterized extensively. The next stage in this area is to predict functional interplay between transporters and drug-metabolizing enzymes to determine the $CL_{int,H}$ of NCEs based on the extended clearance concept (Varma and El-Kattan, 2016). In addition, basolateral and canalicular efflux transporters determine the fate of metabolites formed in the liver, and enzyme–efflux transporter interplay has also been considered for metabolites.

As SCHs maintain metabolic activities and can form a canalicular network, they can be used to investigate the interplay between uptake/efflux transporters and drug-metabolizing enzymes. Indeed, SCHs allow mimicry of species-specific disposition of a parent drug and metabolites in vivo. SCHs are also useful for clinically relevant prediction of DDIs caused by a parent drug and/or metabolite. In addition, mathematical modeling of hepatic disposition of drugs and metabolites in SCHs provides considerable information for mechanistic and quantitative understanding of the event and incorporation into physiologically based pharmacokinetics model simulations.

Tissue engineering is a growing research field, and a number of technologies and newly advanced culture systems, such as cocultures with fibroblasts, have been provided to better reflect in vivo situations (Tetsuka et al., 2017). For instance, HepatoPac, one of the most famous coculture models, has demonstrated longer-term stable expression of drug-metabolizing enzymes as well as basolateral uptake and canalicular efflux transporters (Ramsden et al., 2014; Li et al., 2017). This and other newly developed culture systems may overcome the disadvantage of SCHs; however, a great advantage still exists with SCHs as they have a longer research history for bile canaliculi formation and excretion. Further work will be expected to expand the utility and applicability of hepatocyte-based data including SCHs and other advanced models.

Authorship Contributions

Participated in research design: Matsunaga, Fukuchi, Imawaka, Tamai.

Wrote or contributed to the writing of the manuscript: Matsunaga, Fukuchi, Imawaka, Tamai.

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