Toxicology and Applied Pharmacology 274 (2014) 124–136

Contents lists available at ScienceDirect

Toxicology and Applied Pharmacology

journal homepage: www.elsevier.com/locate/ytaap

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

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ARTICLE INFO

Article history:
Received 24 August 2013
Revised 25 October 2013
Accepted 29 October 2013
Available online 7 November 2013

Keywords:
Drug-induced cholestasis
Bile acids
Sandwich-cultured hepatocytes
Bile salt export pump
Urea assay
Safety margin

ABSTRACT

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

Introduction

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

BA homeostasis is maintained by synchronized activity of different enzymes and transport proteins. Primary BAs (cholic acid and chenodeoxycholic acid) are synthesized from cholesterol in hepatocytes. The synthesized unconjugated BAs (e.g. cholic acid, chenodeoxycholic acid) are conjugated with either glycine or taurine and are excreted into the bile canaliculi by the bile salt export pump (BSEP/Bsep, *ABCB11*/*Abcb11*), an ATP-dependent efflux transporter. Conjugated and unconjugated BAs are further sulfated or glucuronidated in the liver. Sulfated and glucuronidated BAs are transported into the bile canaliculi by multidrug resistance associated protein-2 (MRP2/Mrp2, *ABCC2*/*Abcc2*). Multidrug resistance protein-3 (MDR3/Mdr2, *ABCB4*/*Abcb4*) is a floppase that is involved in translocation of phosphatidylcholine from the inner to the outer bilayer of the bile canalicular membrane. In the bile duct, BAs form mixed micelles after associating with cholesterol and phophatidylcholine. The mixed micelles protect the bile duct surface from the detergent effects of the BAs (Elferink and Paulusma, 2007). In the intestine secondary BAs (deoxycholic acid, lithocholic acid, and ursodeoxycholic acid) are formed by the action of intestinal flora. BAs are taken up by the enterocytes via the apical sodium-dependent bile acid transporter (ASBT/Asbt; *SLC10A2*/*Slc10a2*). Heteromeric organic solute transporters OSTα–β, localized in the basolateral membrane of the enterocytes, effluxes the BAs to the portal circulation (Dawson et al.,

Abbreviations: ALT, alanine aminotransferase; ALP, aspartate aminotransferase; BAs, bile acids; BSEP/Bsep, bile salt export pump (human/rat); DICI, drug-induced cholestasis index; DMSO, dimethyl sulfoxide; TCA, taurocholic acid; CA, cholic acid; FBS, fetal bovine serum; GCDCA, glycochenodeoxycholic acid; GDCA, glycodeoxycholic acid; HBSS, Hanks' balanced salt solution; NOAEL, no observed adverse effect level; NTCP/Ntcp, sodium taurocholate cotransporting polypeptide (human/rat); PBS, phosphate buffered saline; SCH, sandwich-cultured hepatocytes; SCRH, sandwich-cultured rat hepatocytes; SCHH, sandwich-cultured human hepatocytes; SM, safety margin; ULN, upper limit of normal.

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http://dx.doi.org/10.1016/j.taap.2013.10.032

2009; Rao et al., 2008). BAs are taken up from the portal blood into the hepatocytes by sodium taurocholate co-transporting polypeptide (NTCP/Ntcp; *SLC10A1*/*Slc10a1*) and by organic anion transporting polypeptides (OATP/Oatp; *SLCO*/*Slco*). The hepatic uptake of unconjugated BAs is mainly mediated by OATP1B1/Oatp1b2 (*SLCO1B1*/*Slco1b2*) while uptake of conjugated BAs is mostly carried out by NTCP (Csanaky et al., 2011; Dawson et al., 2009; Xiang et al., 2009). Multidrug resistance associated protein-3 and -4 (MRP3/4 and Mrp3/4; *ABCC3/4* and *Abcc3/4*) are the two transporters involved in basolateral (=sinusoidal) efflux of BAs. They are upregulated in cholestatic conditions, when the canalicular efflux of BAs is compromised (Alrefai and Gill, 2007; Bohan and Boyer, 2002).

Disturbances in the normal physiological function of the transporters and enzymes involved in BA homeostasis may lead to cholestasis. Altered enzyme/transporter function may originate from genetic mutations in transporters and enzymes and or external factors such as infections, inflammation, or physical obstruction of the common bile duct, as in the case of gall bladder stones (Epstein et al., 1998; Wagner et al., 2009). Depending on the underlying cause, distinction is made between intra- or extra-hepatic cholestasis. Progressive familial intrahepatic cholestasis (PFIC), benign recurrent intrahepatic cholestasis (BRIC), vanishing bile duct syndrome are some of the forms of intrahepatic cholestasis where modulation of functions of BSEP, MRP2, MDR3 are implicated (Pauli-Magnus and Meier, 2006). However cholestasis can occur from changes in a wide variety of proteins as listed previously (Balistreri et al., 2005).

Increased risk of cholestasis has been reported with certain drug therapies (Stieger et al., 2000). Drug-induced cholestasis has led to the market-withdrawal of troglitazone and nefazodone, while a safety warning has been added to the label of other drugs such as bosentan (Fattinger et al., 2001; Funk et al., 2001). Follow-up studies with these compounds have demonstrated (Marion et al., 2007) inhibition of BSEP by these drugs, leading to intracellular accumulation of BAs and subsequent liver toxicity (Marion et al., 2007; Stieger et al., 2000). The putative role of disturbed BA homeostasis in different forms of hepatotoxicity has recently been substantiated by an untargeted metabolomics study with different hepatotoxicants. The study of Yamazaki et al. (2013) showed that the elevation of BAs in plasma and urine of rats is often one of the early events in drug-induced hepatotoxicity (Yamazaki et al., 2013). The relationship between alteration in glycine-conjugated BA levels and the in vitro toxicity of exogenously administered primary BAs in SCH has been demonstrated as well (Chatterjee et al., in press).

Given the multiplicity and complexity of mechanisms underlying drug-induced cholestasis, early detection of corresponding safety issues during drug development remains highly challenging. Animal models of drug-induced cholestasis can provide valuable mechanistic insights about the progression of cholestatic diseases. However, the animal models suffer from several inherent differences with the human situation such as: (i) BA pools in humans and rodents are qualitatively and quantitatively distinct (Setchell et al., 1997), (ii) the quantitatively major BAs in rodents (taurine conjugated) are more hydrophilic and less toxic than the major BAs present in human (glycine conjugated) (Rodriguez-Garay, 2003). Not surprisingly, with the current biochemical and histological markers only 50% of the clinical cases of liver toxicity are detected in preclinical animal models (Olson et al., 2000). In addition, the in vitro testing models using human hepatocytes detected only 50–60% cases (drugs and drug candidates) of drug-induced liver injury (Xu et al., 2008).

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

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

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

Material and methods

Materials. Williams' E Medium (WEM), L-glutamine, penicillin–streptomycin mixture (contains 10,000 IU/ml potassium penicillin and 10,000 μg/ml of streptomycin sulfate), Dulbecco's modified eagle's medium (DMEM), fetal bovine serum (FBS), Hanks' balanced salt solution (HBSS) (referred to as 'standard buffer' when pH adjusted to 7.4), Phosphate Buffered Saline (PBS; 1× and 10×), and Trypan blue solution (0.4%) were purchased from Lonza Verviers SPRL (Verviers, Belgium). ITS + ™ Premix (contains insulin 6.25 mg/l, transferin 6.25 mg/l, selenious acid 6.25 mg/l, bovine serum albumin 1.25 g/l and linoleic acid 5.35 mg/l) was purchased from BD Biosciences (Erembodegem, Belgium). Sulfuric acid (95–97%) was purchased from Chem-Lab NV (Zedelgem, Belgium). All BAs, collagenase type IV (from Clostridium histolyticum), ECM gel (from Engelbreth-Holm-Swarm murine sarcoma), recombinant human insulin, dexamethasone, urea, diacetyl monoxime, thiosemicarbazide, iron (III) chloride hexahydrate, ortho-phosphoric acid, ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA), Dulbecco's modified eagle's medium 10× (DMEM 10×), and 5(6)-carboxy-2′,7′-dichlorofluorescein diacetate (CDFDA) were purchased from Sigma-Aldrich (Schnelldorf, Germany). Cyclosporin A, troglitazone, and bosentan were purchased from Sequoia Research Products Ltd, UK. HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) was purchased from MP Biochemical (Illkirch, France). 48 and 24-well sterile cell culture plates were purchased from Greiner Bio-One BVBA (Wemmel, Belgium). Thermostable 96-well plates (for urea assay) were kindly provided by Greiner Bio-One BVBA (Wemmel,