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Differential inhibitory effect of cyclosporin A and bosentan on taurocholate uptake in human and rat hepatocytes as a function of culturing time

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ABSTRACT

Bile salt transport across hepatocytes requires a coordinate action of transporters, which is thought to be a target for drug-induced cholestasis. Hepatocytes provide the most competent *in vitro* model to predict transporter-related toxic drug effects. The aim of this study was to show a correlation between inhibitory potential of drugs and the change of rate, as well as of the active to passive ratio of taurocholate uptake in these cells. In rat hepatocytes, along with a significant decrease of uptake (86.4% by 72 h), and the shift of saturable/unsaturable transport (from 92/8 to 55/45 in a 24–72 h time interval), the efficacy of taurocholate uptake inhibition was highly reduced (IC_{50} cyclosporin A 3.9 to >100 μM, and bosentan 9.1–49.8 μM at 1 and 72 h, respectively). In contrast, 5-day-old human hepatocytes preserved 70% of their taurocholate uptake capacity with a 2-fold higher active than passive transport, which resulted in a more efficient inhibition by drugs (IC_{50} cyclosporin A, 2.4 to ~10 μM and bosentan 28.9–45.5 μM at 1 h and 5 days, respectively). Our results support that reliable drug interaction studies might be performed in 5-day-old human hepatocyte cultures, while experiments using rat hepatocytes at more than 24 h after seeding will highly underestimate the probability of drug interaction.

1. Introduction

The first step in drug elimination process is hepatic uptake across the basolateral membrane, thus hepatic uptake rate might be one of the determinants of drug disposition (Szakács et al., 2008; Kusuhara and Sugiyama, 2009). Drug interactions resulting in altered expression and/or function of uptake transporters might change the elimination of endogenous compounds and pharmacokinetic properties of xenobiotics leading to toxicity (Koepsell et al., 2007; Treiber et al., 2007; Fahrmayr et al., 2010). One frequently cited example is that of modulation of hepatobiliary transport of bile salts and subsequent alteration of bile acid homeostasis, which may be one of the causes of drug-induced hepatotoxicity. Although bile salt export pump (BSEP) has been the major focus of transport inhibition as a mechanism of drug-induced liver injury, inhibition of basolateral hepatic uptake of bile salts by many of cholestatic drugs has also been shown (Fattinger et al., 2001; Kemp et al., 2005; Mita et al., 2006). Taurocholate (TC) is a commonly used substrate for modeling drug-transporter interactions affecting bile acid homeostasis. Hepatic uptake of TC is mediated by both Na(+)-taurocholate co-transporting polypeptide (NTCP) and organic anion transporting polypeptide (OATPs) systems. The OATP proteins, however, contribute substantially less to the overall uptake process both in humans and rodents, than NTCP does (Kullak-Ublick et al., 2004; Leslie et al., 2007).

TC uptake occurs via unsaturable passive, and saturable transporter-mediated processes, simultaneously. The ratio of these processes depends on the passive permeability of a compound, and the expression and/or activity of transporters involved (Hallifax and Houston, 2007; Shugarts and Benet, 2009). A significant intracellular concentration difference may exist as a result of reduced uptake activity caused by processes, such as drug–drug interaction, impaired synthesis or trafficking of the transporter, cholestasis etc. (Jigorel et al., 2006; Geier et al., 2007), which highly influence the rate of metabolism and efflux of compounds and metabolites. Thus, the use of *in vitro* data for quantitative prediction of drug elimination and drug–drug interaction requires the estimation of the ratio of active to passive components of transport in an *in vitro* system used (Poirier et al., 2008).

Primary hepatocytes, as well as cell lines over-expressing individual transporters, are widely used to study drug–transporter

Abbreviations: NTCP, Na(+)-taurocholate co-transporting polypeptide; BSEP, bile salt export pump; OATP, organic anion transporting polypeptide; TC, taurocholate; BSP, sulfobromophthaleine; HBSS, Hanks' Balanced Salt Solution.

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interaction processes. Cell lines over-expressing a single transporter provide useful models to elucidate whether a drug is transported by a given transporter, and to determine the kinetic parameters of the transport (Kopplow et al., 2005; Treiber et al., 2007). Hepatocytes provide the most physiologically relevant model for measuring qualitative and quantitative aspects of hepatic clearance, as they contain an almost full set of drug-metabolizing enzymes and drug transporters (Soars et al., 2007) representing a more complex system than cell lines over-expressing a selected transporter (Yamada et al., 2007). Hepatocyte-based assays are widely used to predict transport processes quantitatively, in spite of difficulties in estimating the role of individual transporters, since most substrates are taken up by more than one transporters and often interfere with others (Kemp et al., 2005; Abe et al., 2008). At the same time, there is a basic limitation to primary hepatocyte-based assays in that they exhibit early phenotypic alterations.

It is well documented that due to dedifferentiation processes, hepatocytes rapidly loose their uptake transporter activity in culture, moreover, their efflux transporter expression changes as well. These processes can result in different uptake rates, active/passive uptake ratio in parallel with altered efflux rates leading to highly diverse intracellular drug concentrations when studied at different times of culturing (Liu et al., 1998; Borlak and Klutcka, 2004; Jigorel et al., 2005; Bow et al., 2008). The outcome of drug interaction studies as a function of culturing time and configuration of hepatocyte cultures, however, has been less studied.

The aim of this study was to investigate the shift of inhibitory potential of drugs in function of the rate, and active-to-passive ratio of TC uptake related to culturing time. Because of known interspecies differences in survival and preservation of differentiated liver functions in cultured cells (Jigorel et al., 2005), this work was conducted with both human and rat hepatocytes. Cyclosporin A and bosentan, two well characterized TC uptake inhibitors, were used in drug interaction studies. Both are known to cause cholestasis in humans, partly due to modulation of bile salt transport processes.

2. Methods

2.1. Materials

[^{3}H] taurocholate was obtained from American Radiolabeled Chemicals Inc (St Louis, MO), taurocholate, cyclosporin A, ketoconazole, glibenclamide, troglitazone, sulfobromophthaleine, type IV collagenase, all cell culture media and reagents were purchased from Sigma–Aldrich (Budapest, Hungary). Bosentan was purchased from Sequoia Research Products Ltd. (Pangbourne, UK). Twenty-four well plates were obtained from Greiner Bio-One (Mosonmagyarovar, Hungary). All other chemicals were of analytical grade.

2.2. Preparation and culture of primary human and rat hepatocytes

Human hepatocytes were obtained from 10 donors (6 males, 4 females) undergoing hepatic resection at Uzsoki Hospital (Budapest, Hungary). All of the patients were operated with adenocarcinoma metastasis hepatis, and their age was 52–71. Cells were prepared by a three-step perfusion of histologically normal liver fragments as described previously (Lengyel et al., 2005). Permission of the Local Research Ethics Committee was obtained to use human tissues. Rat hepatocytes were prepared from male Wistar rats (200–250 g) (Charles River, Budapest). The isolation and culture of rat hepatocytes were performed as described previously (Lengyel et al., 2005). All procedures were approved by the Institutional Animal Care and Use Committee. Cell viability (>90%) was determined by trypan blue exclusion. Hepatocytes were plated at a density of 1.9×10^5 cells/cm^2 in 24-well plates precoated with rat tail collagen in Williams Medium E containing 5% of fetal calf serum, 100 nM insulin, 0.1 mg/ml gentamicin, 30 nM Na_2SeO_3, and 0.1 μM dexamethasone. Calf serum was present for the first 24 h, then omitted. Cells were maintained at 37 °C in a humidified atmosphere of 95% air-5% CO_2. 1 h after plating, and every day thereafter the medium was changed to Williams Medium E supplemented with glucagon, insulin, gentamicin, dexamethasone, Na_2SeO_3. To achieve sandwich configuration, cells were overlaid with matrigel basement membrane matrix at a concentration of 0.25 mg/ml in 0.4 ml of ice-cold Williams Medium E supplemented with glucagon, insulin, gentamicin, dexamethasone and Na_2SeO_3 at 24 h after plating.

2.3. TC uptake experiments

For uptake experiments, rat hepatocytes were used at 1, 24, 48, 72 and 96 h, while human hepatocytes at 1, 24, 120, 168 h after plating, respectively. Experiments were performed as described previously (Jemnitz et al., 2010). Briefly, wells were washed at 37 °C with Hanks' Balanced Salt Solution (HBSS) buffer. The uptake experiment was started by the addition of 400 μl pre-warmed HBSS solution containing 1 μM of ^{3}H TC and lasted for 10, 20, 30, 60, 120, and 300 s at 37 °C. Uptake was terminated by removal of the substrate-containing buffer, and wells were rinsed three times with ice-cold HBSS. Cells were lysed with 0.5 ml of 0.5% Triton X 100 in HBSS by shaking for 20 min at room temperature. An aliquot of lysate was analyzed by liquid scintillation. The protein content of lysates was determined by the method of Lowry et al. (1951). Assays were run using four wells in one set. All experiments were carried out with hepatocytes from three independent cell preparations.

2.4. Kinetic and drug interaction experiments

Kinetic experiments were carried out as described above. ^{3}H TC was incubated at six different (1–300 μM) concentrations for 30 s. Four wells were used for each concentration, and kinetic parameters were calculated from experiments with hepatocytes from three independent preparations. For drug interaction experiments 1 μM of ^{3}H TC was incubated in the presence of the inhibitors, cyclosporin A, bosentan, glibenclamide, ketoconazole, troglitazone, sulphobromophthalein (BSP) (1–100 μM), or the vehicle (DMSO, 0.1%), respectively. The uptake lasted for 30 s, and was terminated by removal of the TC and the modulator-containing medium. Assays were run using four wells as one set. All experiments were carried out with hepatocytes from three independent cell preparations.

2.5. Data analysis

The total amount of TC taken up by hepatocytes was determined in cell lysates and was expressed as pmol/mg prot.

The active and passive component of the uptake, and the kinetic parameters were calculated using the following equation (Yamada et al., 2007; Ye et al., 2008):

$$V = \frac{V_{max} \times S}{K_m + S} + P_{dif} \times S \quad (1)$$

where V is the uptake velocity of the substrate (pmol/min/mg protein), S is the substrate concentration in the medium (μM), K_m is the Michaelis constant (μM), V_{max} is the maximum uptake rate (pmol/min/mg protein), and P_{dif} is the passive, nonsaturable uptake clearance (μl/min/mg protein). The highest two concentrations of uptake experiments were set to be 5–10 times higher than K_m. At this concentration, the active process is saturated, so the uptake rate vs.