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Human and rat precision-cut intestinal slices as *ex vivo* models to study bile acid uptake by the apical sodium-dependent bile acid transporter

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ABSTRACT

The apical sodium-dependent bile acid transporter (ASBT) is the primary transporter for the uptake of bile acids in the small intestine. It is localized on the apical membrane of the ileal enterocytes and is known for its high capacity and affinity for taurocholic acid *in vitro*. However, less is known about its activity towards taurocholic acid and other bile acids *in vivo* due to the lack of a suitable model. The aim of this study was to validate precision-cut intestinal slices (PCIS) of rat and human intestine as *ex vivo* model to study the activity of ASBT-mediated transport, and subsequently to study regional and interspecies differences in ASBT function. PCIS maintain the natural cell polarization and communication, expression of transporters and metabolizing enzymes. Therefore this model is expected to be more relevant to the *in vivo* situation than *in vitro* cell culture models. PCIS of human and rat ileum were prepared and incubated with 0.04–2.00 mM taurocholic acid, deoxycholic acid and cholic acid, respectively at 4 and 37 °C. In this study, the respective contribution of active uptake appeared to be higher for taurocholic acid whereas the passive diffusion was higher for deoxycholic acid and cholic acid. Furthermore, the rank order of calculated (apparent) K_m and V_{max} for these bile acids is in line with literature reports. The active uptake of taurocholic acid in rat PCIS could be inhibited partly by simvastatin and fluvastatin and was fully inhibited by the specific inhibitor GSK2299027B, supporting the involvement of rat ASBT as uptake transporter. In both species, the ASBT-mediated active uptake of bile acids was observed only in the ileum whereas only passive diffusion was observed in the jejunum and colon. In addition, ASBT activity was higher in the human ileum compared to the rat ileum. In the future rat and human PCIS can be used to study the uptake of new ASBT substrates and as a screening method for potential ASBT inhibitors.

1. Introduction

Major routes for the uptake and excretion of endogenous and exogenous compounds are passive diffusion and active transport by uptake and efflux transporters. In the intestine multiple transporters are expressed on both the apical and basolateral membrane of the enterocytes. The apical uptake transporters in the small intestine play important roles in facilitating the influx of various nutrients, bile acids, drugs and their metabolites that are substrates of these uptake transporters (Ayrton and Morgan, 2001). The apical sodium-dependent bile acid transporter (ASBT), a member of the solute carrier (SLC) super-family encoded by the *SLC10A2* gene, is such an uptake transporter for bile acids (Dawson et al., 2009).

The intestinal absorption of bile acids has long been known as a remarkably efficient process, leading to reabsorption of at least 95% of the bile acids that are secreted in human bile (Kullak-Ublick et al., 2004). The majority of bile acids is taken up in the small intestine and only approximately 5% (or approximately 0.5 g/d) of the intestinal bile

Abbreviations: ASBT, apical sodium-dependent bile acid transporter; DMSO, dimethyl sulfoxide; NTCP, Na^+-taurocholate cotransporting polypeptide; OATP, organic anion transporting polypeptide; PBS, phosphate buffered saline; PCIS, precision-cut intestinal slices; PPPD, pancreaticoduodenectomy; SLC, solute carrier; GSK20, GSK 2299027B, {[(3*R*,5*R*)-3-Butyl-3-ethyl-7-(methyloxy)-1,1-dioxido-5-phenyl-2,3,4,5-tetrahydro-1,4-benzothiazepin-8-yl]methyl}-phosphonic Acid Hydrochloride Salt

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acids reaches the colon. Absorption of bile acids occurs *via* passive and transporter-mediated processes. While passive transport occurs along whole the length of the small intestine, transporter-mediated uptake occurs mainly in the terminal ileum (Martínez-Augustin and de Medina, 2008). After uptake into the enterocytes, bile acids are bound to Bile Acid Binding Protein (BABP) and excreted at the basolateral side into the portal blood by organic solute transporter (OST) α/β and multidrug resistance-associated protein (MRP) 1 and 3. ASBT has been identified as the main actor for the active process and is responsible for the majority of uptake of conjugated bile acids (Dawson, 2011). In human small intestine ASBT is solely localized on the apical membrane of the ileum whereas rat ASBT is primarily expressed on the apical membrane of the ileum but also expressed in the caecum (Shneider et al., 1995). ASBT has been shown to exhibit a high capacity transport of conjugated bile acids from the intestinal lumen into ileal epithelium (Schiff et al., 1972; Sun et al., 1998; Kramer et al., 1999; Shneider, 2001; Hagenbuch and Dawson, 2004). Both conjugated and unconjugated bile acids are transported by ASBT but the former have higher affinity for this transporter. Thus, conjugated bile acids, which are the most abundant and more hydrophilic (Sjovall, 1959; Northfield and McColl, 1973), are more efficiently transported by ASBT (Alrefai and Gill, 2007), compared to the more hydrophobic unconjugated bile acids. Furthermore, dihydroxy bile acids (chenodeoxycholic acid and deoxycholic acid) generally have a higher affinity for ASBT than the trihydroxy bile acids (cholic acid and taurocholic acid) (Schiff et al., 1972; Craddock et al., 1998; Balakrishnan et al., 2006). Malfunction of ASBT can lead to inflammatory bowel disease, constipation, diarrhea and Alagille syndrome (Shneider, 2001; Chen et al., 2012; Acosta and Camilleri, 2014).

Due to its critical role in bile acid homeostasis, ASBT has been an attractive target for drug development since it was discovered and identified, and it was hypothesized that the regulation of ASBT activity could serve as a treatment for several intestinal diseases, type 2 diabetes and hypercholesterolemia (Root et al., 2002; Wu et al., 2013). For example, elobixibat, a selective and partial ASBT inhibitor, has been developed for treatment of chronic constipation and constipation-predominant irritable bowel syndrome, and GSK2330672, a highly potent ASBT inhibitor, for the treatment of type 2 diabetes, based on the influence of bile acids on glucose homeostasis, influencing glucagon-like peptide secretion. Both compounds are now in clinical trials (Wu et al., 2013; Rudling et al., 2015). Several drugs have been identified as ASBT inhibitors, such as simvastatin and fluvastatin. Moreover, ASBT has been investigated as a transporter for uptake of drug-bile acid conjugates (Dawson, 2011), where bile acids serve as “Trojan Horses” by coupling a drug molecule to a bile acid (Balakrishnan and Polli, 2006) in order to increase the oral availability of such a drug.

Multiple tools have been developed to study bile acid uptake by ASBT. ASBT function has been studied *in vitro* with ASBT-expressing (CHO, COS, HEK and MDCK) cells, oocytes, and membrane vesicles derived from those cells (Baringhaus et al., 1999; Balakrishnan et al., 2006; Zamek-Gliszczynski et al., 2013). The use of wild type or genetically modified animals and *in situ* perfusion models has also been reported (Baringhaus et al., 1999; Chen et al., 2006). However, these *in vitro* models differ in many aspects from the conditions *in vivo*, while animal experiments are costly and a large number of animals is required for statistical evaluation as it allows only one experimental condition per animal. Precision-cut intestinal slices (PCIS) are a well-established *ex vivo* model to investigate drug metabolism, toxicity, and recently efflux transport in the intestine, allowing hundreds of tests per animal, thereby contributing to the reduction in animal use. Moreover this technique can be applied to human intestine with a tiny piece of tissue, which is obtained as medical waste after surgical resections (Li et al., 2015, 2016, 2017a, 2017b). In addition, each slice contains all types of cells in the tissue in their natural environment, and consequently the natural cell polarization and expressions of transporters and metabolizing enzymes are maintained (van de Kerkhof et al., 2005; van de Kerkhof et al., 2008; de Graaf et al., 2010). As a result, besides ASBT, also other bile acids transport mechanisms, such as uptake *via* organic anion-transporting polypeptide (OATP) 1A2, apical efflux *via* MRP2, basolateral efflux *via* OSTa/b, MRP1 and MRP3, are functional in the enterocytes in the PCIS. Therefore, the overall bile acid uptake measured in PCIS might better represent the physiological situation than in cells where only ASBT is over-expressed. In addition PCIS can reflect the differences along the intestine, in contrast to Caco2 cells that represent at best only one small region. By careful design of the experiments, the contribution of ASBT excluding the interferences from other bile acid uptake and excretion pathways can be assessed. Thus, PCIS are expected to be a suitable *ex vivo* model to study uptake by ASBT in the intestine.

Therefore, the aims of this study were: (1) to validate rat and human PCIS as models to study the activity of ASBT; (2) to map regional differences in bile acid uptake; (3) to investigate species differences in bile acid uptake. In order to distinguish the passive and active uptake of bile acids, the incubations were performed at 4 and 37 °C in parallel. It is generally accepted that active transport (MRPs are directly dependent on energy of ATP and ASBT and OATP are secondary active transporters) does not function at 4 °C while passive transport processes are not highly temperature dependent. At 37 °C, both active and passive transport are fully functional. Thus, subtraction of the uptake at 4 °C from that at 37 °C will largely represent the active uptake, although some overestimation due to a small reduction of passive uptake at low temperatures cannot be excluded.

Three different bile acids, *i.e.* cholic acid, taurocholic acid and deoxycholic acid, were used in this study because of their differences in physicochemical properties regarding the number of hydroxyl groups. Cholic acid and taurocholic acid are trihydroxy bile acids while deoxycholic acid is a dihydroxy bile acid. Furthermore, they represent three categories of bile acids: a primary bile acid (cholic acid), a secondary bile acid (deoxycholic acid), and a conjugated bile acid (taurocholic acid: a conjugate of cholic acid and taurine) (Hofmann and Hagey, 2008; Stamp and Jenkins, 2008). Moreover, the inhibition of uptake of taurocholic acid was studied in rat PCIS using the specific and potent ASBT inhibitor GSK 2299027B (GSK20) (Wu et al., 2013) and by two drugs known to inhibit ASBT, fluvastatin and simvastatin (Dong et al., 2013; Zheng et al., 2009).

2. Materials and methods

2.1. Chemicals

Sodium taurocholate hydrate, sodium deoxycholate, agarose (low gelling temperature, type VII-A), Trizma® hydrochloride (Tris) and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich Co. LLC (USA). Williams Medium E with glutamax-I, fungizone® antimycotic (amphotericin B) and gentamicin were purchased from Gibco® (UK). D (+)-Glucose monohydrate, sodium hydroxide, EDTA together with sodium chloride and cholic acid sodium salt were derived from Merck Millipore (USA). Hepes was purchased from MP Biomedicals (Germany), methanol and ethanol absolute were from VWR Chemicals (USA). Both hematoxylin and eosin were purchased from Klinipath (the Netherlands).

Bile acid stock solutions were prepared in DMSO. Simvastatin was a gift from the Hospital Pharmacy of the University Medical Centre Groningen and fluvastatin were obtained from Sigma Aldrich Co. LLC (USA), GSK 2299027 (GSK20) {[(3*R*,5*R*)-3-Butyl-3-ethyl-7-(methyloxy)-1,1-dioxido-5-phenyl-2,3,4,5-tetrahydro-1,4-benzothiazepin-8-yl] methyl}-phosphonic acid hydrochloride salt, was a kind gift of Jon L. Collins, PhD (GlaxoSmithKline,R&D, US Discovery Partnerships with Academia, Research Triangle Park, NC. 27709).

2.2. Animals

All experiments were approved by the Animal Ethical Committee of

Table 1
Characteristics of the human intestine donors.

Patient No.	Gender	Age	Region	Surgical Procedure
1	F	66	ileum & colon	hemicolectomy
2	M	64	jejunum	PPPD
3	F	53	colon	hemicolectomy
4	F	50	jejunum	PPPD
5	M	54	jejunum	PPPD
6	M	76	ileum & colon	hemicolectomy
7	M	63	colon	hemicolectomy
8	M	51	ileum	hemicolectomy

the University of Groningen. Male Wistar (HsdCpb: WU) rats with a body weight ranging between 300 and 350 g were obtained from Harlan (Horst, the Netherlands). The rats were housed in a temperature- and humidity controlled room and fed *ad libitum* for at least 7 days before the experiment.

2.3. Human intestinal tissue

The use of human tissue was approved by the Medical Ethical Committee of the University Medical Centre Groningen. The pieces of jejunum were derived from patients undergoing a pylorus-preserving pancreaticoduodenectomy (PPPD). Ileum and colon were obtained from patients undergoing a hemicolectomy. In some cases, during this procedure, a piece of colon and a piece of ileum were collected from the same patient. The characteristics of the donors are listed in Table 1. Immediately after resection the human tissue was stored in carbogenated ice-cold Krebs-Henseleit buffer (supplemented with 10 mM Hepes and 25 mM D-glucose, pH 7.4) and transported to the laboratory within 20 min.

2.4. Preparation and incubation of rat and human PCIS

Precision-cut intestinal slices from human and rat were prepared as previously described (de Graaf et al., 2010; Li et al., 2015). Rats were anaesthetized with isoflurane/O_2 and exsanguinated *via* the aorta. The whole intestine was removed from the rat and directly stored at ice-cold carbogenated Krebs-Henseleit buffer. Rat intestine was divided into the duodenum, jejunum, ileum and colon. The first 2 to 12 cm distal from the pylorus was used as duodenum and the part between 20 and 40 cm as jejunum. The last 10 cm proximal from the ileocecal junction was used as ileum and the part after ileocecal junction as colon. In case of human intestine, the muscle layer was removed and the mucosa layer was cut into segments of 10 × 20 mm before embedding. Both human and rat intestine were embedded using a tissue embedding unit (Alabama R&D, USA) into agarose solution (3% agarose in 0.9% NaCl) at 37 °C, which solidified on ice. The resulting cores were sliced using the Krumdieck tissue slicer (Alabama R&D, USA) into precision-cut slices with a wet weight of 3–4 mg representing a thickness of 300–400 µm. After removal of the agarose with a spatula, the individual slices were pre-incubated for 30 min in a 12-well culture plate (Greiner Bio-One GmbH, Austria) containing 1.3 ml Williams Medium E supplemented with fungizone (2.5 µg/ml), gentamicin (50 µg/ml) and D(+)-glucose (25 mM). Depending on the aim of the experiment, the 12-well culture plates with slices were placed in plastic boxes in a cabinet with carbogen (95% O_2/5% CO_2), either at 37 °C or at 4 °C (on ice). A reciprocal shaker was used at approximately 90 times per minute (Reciprocating Shaker 3018, Gesellschaft für Labortechnik GmbH, Germany).

2.5. Intracellular ATP

The viability of the slices was evaluated by measuring the intracellular ATP content (van de Kerkhof et al., 2008). After slicing, three slices were directly snap-frozen in 1 ml sonification solution (ethanol 70% v/v containing 2 mM EDTA, pH 10.9) in liquid N_2 as control (fresh slices). Furthermore, the ATP content of control slices ($t = 0$) was determined after 30 min of pre-incubation without bile acids. To test the toxicity of the bile acids, the ATP content was measured after incubation with or without bile acids at concentrations up to 2.00 mM, for 60 min for taurocholic and cholic acid or for 10 min for deoxycholic acid, as deoxycholic acid was toxic for slices at all tested concentrations after 60 min of incubation. The samples were stored at −80 °C until the ATP determination. The intracellular ATP content of the samples was assessed using the ATP Bioluminescence Assay Kit CLS II (Roche Applied Sciences, Germany) and a Spectramax micro-plate reader (Molecular Devices, USA).

2.6. Evaluation of slice histomorphology

Rat PCIS were collected after the exposure to bile acids as described above and fixed in 4% (v/v) formalin for 24 h at 4 °C. After 24 h the slices were transferred into 70% ethanol (v/v) and stored another 24 h at 4 °C, followed by dehydration by submersion in solutions with increasing ethanol percentage up to 100%. After dehydration, PCIS were embedded in paraffin and 4 µm sections were cut. Hematoxylin and eosin staining was performed as described previously (de Graaf et al., 2007).

2.7. Kinetics of bile acid uptake

The kinetics of bile acid uptake were assessed at 37 and 4 °C (Zamek-Gliszczynski et al., 2013). Active uptake was estimated by subtracting the passive uptake at 4 °C from the total uptake at 37 °C (Lave et al., 2008).

After 30 min of pre-incubation, taurocholic acid, cholic acid or deoxycholic acid was added, and the slices were harvested after 0, 5, 10, 15, 30 and 60 min. Concentration-dependent uptake was assessed by incubating slices with 0.02 to 2.00 mM bile acid for 10 min. The concentrations used were below the bile acid specific critical micelle concentration, which is approximately 13 mM for cholic acid and 10 mM for both taurocholic acid and deoxycholic acid (Stamp and Jenkins, 2008). After the incubation, the slices were washed three times with cold PBS and stored at −20 °C until bile acid measurement.

2.8. Inhibition of bile acid uptake by GSK20, fluvastatin and simvastatin

First, the toxicity of GSK20, fluvastatin and simvastatin was determined by incubating rat PCIS with GSK20 (0.002–0.1 mM), fluvastatin (0.05–0.250 mM) and simvastatin (0.05–0.250 mM), respectively at 37 °C for 1 h. The intracellular ATP content was determined and corrected for the amount of protein. Inhibitory effects were assessed after 30 min of pre-incubation with the inhibitors followed by 10 min incubation with inhibitors and 0.04 mM taurocholic acid. After the incubation, the slices were washed three times with cold PBS and stored at −20 °C until bile acid measurement.

2.9. Bile acid measurement

450 µl of 70% methanol (v/v) was added to the slices and the slices were homogenized for 45 s using a Mini BeadBeater-8 (Biospec, USA). After centrifugation for 15 min at 13,000 rpm (room temperature) 350 µl supernatant was transferred into a clean tube and dried by CentriVap Aqueous Concentrator System (Labconco, USA). The dry residue was reconstituted with 100 µl TRIS-HCl (50 mM, pH 7.6) and 2 µl was added to a 96-well plate. The total bile acid content was then analyzed using the DZ042A-K Total Bile Acids Test Kit (Diazyme, USA). The absorbance at 405 nm was measured every 5 min for 30 min with a Synergy HT Microplate Spectrophotometer (BioTek, USA), and the total bile acid content was calculated based on the calibrators and blank samples provided.

2.10. Analysis of protein content

The remaining pellet after homogenization of the slices for the ATP and bile acid measurement was dried overnight at 37 °C. The dried pellet was dissolved in 200 µl NaOH (5 M) and incubated in water bath at 37 °C for 30 min. Afterwards 800 µl ultrapure water was added to a final concentration of 1 M NaOH and the sample was homogenized for 40 s. The amount of protein was determined using a DC™ Protein Assay (Bio-Rad, USA) with Lumicount microplate luminometer (Packard, USA). The measured amount of ATP and bile acids in the slices were corrected for the protein content.

2.11. Statistical analysis

Experiments were performed with intestinal tissues from at least three different humans and rats. For each condition within an experiment three slices were used and the average value of the three slices was used as the value for that particular condition of the individual human or rat. The results are given as the mean of individual experiments ± SEM. For the analysis of ATP content in rat and human PCIS, one-way ANOVA with a Bonferroni's post-hoc test was used to compare multiple groups with one factor. The level of significance was set at $p < 0.05$. Kinetics parameters of ASBT were calculated and the Michaelis-Menten curves were fitted by GraphPadPrism 5 (GraphPad Inc., USA).

3. Results

3.1. Toxicity of bile acids

As shown in Fig. 1, the ATP content in PCIS remained constant after incubation with 2 mM cholic acid and taurocholic acid up to 1 h, indicating that these bile acids are not toxic under these circumstances. However, the ATP content decreased in a concentration-dependent manner in rat ileum slices incubated with deoxycholic acid for 10 min. A significant decrease in ATP content was found in the slices incubated with 2.00 mM deoxycholic acid (0.8 nmol/mg protein) compared to that in the control slices (3.8 nmol/mg protein), indicating that deoxycholic acid at 2.00 mM decreased the viability of rat PCIS. Human ileum slices incubated with 2.00 mM taurocholic acid for 3 h showed no significant decrease in ATP content compared to the control slices, 5.6 and 4.4 nmol/mg protein, respectively.

For morphological evaluation rat PCIS were incubated in the same manner as the slices for the determination of intracellular ATP. In Fig. 2 morphological sections of slices incubated with cholic acid, taurocholic acid, and deoxycholic acid, respectively, and stained with hematoxylin and eosin are depicted. Sections a–j show no obvious decrease in morphological integrity of the stroma, crypts, muscle layer and the epithelium. A continuous epithelial lining is present with cubic epithelial cells (Sections a–j). In contrast, in both sections k and l epithelial cells are flattened and a discontinuous epithelial lining is visible. A detachment of the muscle layer is seen in slices incubated with 1.00 mM deoxycholic acid (Section k) and slices incubated with 2.00 mM deoxycholic acid show a considerable loss of integrity of the epithelial lining (Section l). This morphological damage is in line with the decrease of intracellular ATP, confirming the loss of viability.

3.2. Regional differences in uptake of taurocholic acid

Uptake of taurocholic acid at a concentration of 0.04 mM was determined at different regions of rat and human intestine. This low concentration was chosen as pilot studies showed that no saturation of ASBT takes place at this concentration. The time-course of uptake in PCIS from different regions for rat and human intestine is shown in Fig. 3. Total uptake of taurocholic acid at 37 °C (*i.e.* both active transport and passive uptake, Fig. 3a and d) was highest in the ileum compared to duodenum, jejunum and colon for both rat and human whereas the passive uptake of taurocholic acid (Fig. 3b and e) was relatively low (0.2–0.6 pmol/mg protein) and equal in all the intestinal regions of rat and human. The active uptake of taurocholic acid by ASBT (Fig. 3c and f) was determined after subtraction of passive taurocholic acid uptake at 4 °C from the total uptake at 37 °C. As shown in Fig. 3c active uptake by rat ASBT only occurred in the ileum and not in the other regions. The same accounts for human PCIS (Fig. 3f). The uptake rate was linear during 15 min (Fig. 3c and f).

3.3. Concentration-dependent uptake of bile acids

Based on the results depicted in Fig. 3c and f, the concentration-dependent ASBT-mediated uptake of taurocholic acid, deoxycholic acid and cholic acid was measured at 10 min with different concentrations ranging from 0.04 to 2.00 mM. The incubation time of 10 min was chosen since it was shown to be within the linear range of uptake, ensuring that the uptake rate was accurately measured (Audus et al., 1996). Fig. 4a and b show a concentration-dependent uptake of cholic acid, taurocholic acid and deoxycholic acid in rat PCIS at 4 and 37 °C, respectively. At 4 °C, bile acid uptake was lower and not saturable, indicating passive diffusion. The uptake of deoxycholic acid at 4 °C was relatively high compared to the uptake of the less lipophilic cholic acid and taurocholic acid. The rate of the active uptake was calculated and showed clear saturable uptake for all bile acids as depicted in Fig. 4c. The apparent K_m of the rat ASBT for the three bile acids, calculated by fitting the data to the Michaelis-Menten equation, is shown in Fig. 4c and listed in Table 2, and showed the following rank order: taurocholic acid < deoxycholic acid < cholic acid. The apparent V_{max} of rat ASBT, indicating the capacity of the ASBT-mediated uptake, is higher for deoxycholic acid than for cholic acid and taurocholic acid (Table 2) with the following rank order: deoxycholic acid > taurocholic acid > cholic acid (Fig. 4c). The contribution of rat ASBT to the total uptake was higher for taurocholic acid compared to cholic acid and deoxycholic acid at all concentrations (Fig. 4c), and was up to 87.0% at low concentrations of taurocholic acid and varied from 35.6% (deoxycholic acid) to 56.4% (taurocholic acid) at 2.00 mM.

Human ileum is scarce and therefore only taurocholic acid was used for the characterization of the concentration-dependent uptake of ABST. Total uptake and passive diffusion of taurocholic acid in human

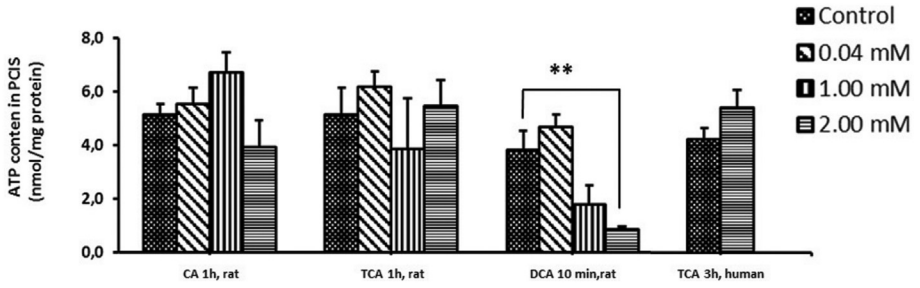

Fig. 1. Effect of bile acids on the viability of PCIS. The ATP content in rat ($n = 3$) and human ($n = 2$) ileum PCIS after the indicated incubation time in absence or presence of cholic acid (CA), taurocholic acid (TCA), and deoxycholic acid (DCA) respectively. The data are depicted as mean ± SEM. One-way ANOVA with Bonferroni's *post hoc* test was used for the comparison between each group incubated with bile acids and the corresponding control group. Statistical difference in intracellular ATP was found between the rat PCIS treated with 2.00 mM deoxycholic acid and the corresponding control slices (**$p < 0.05$).

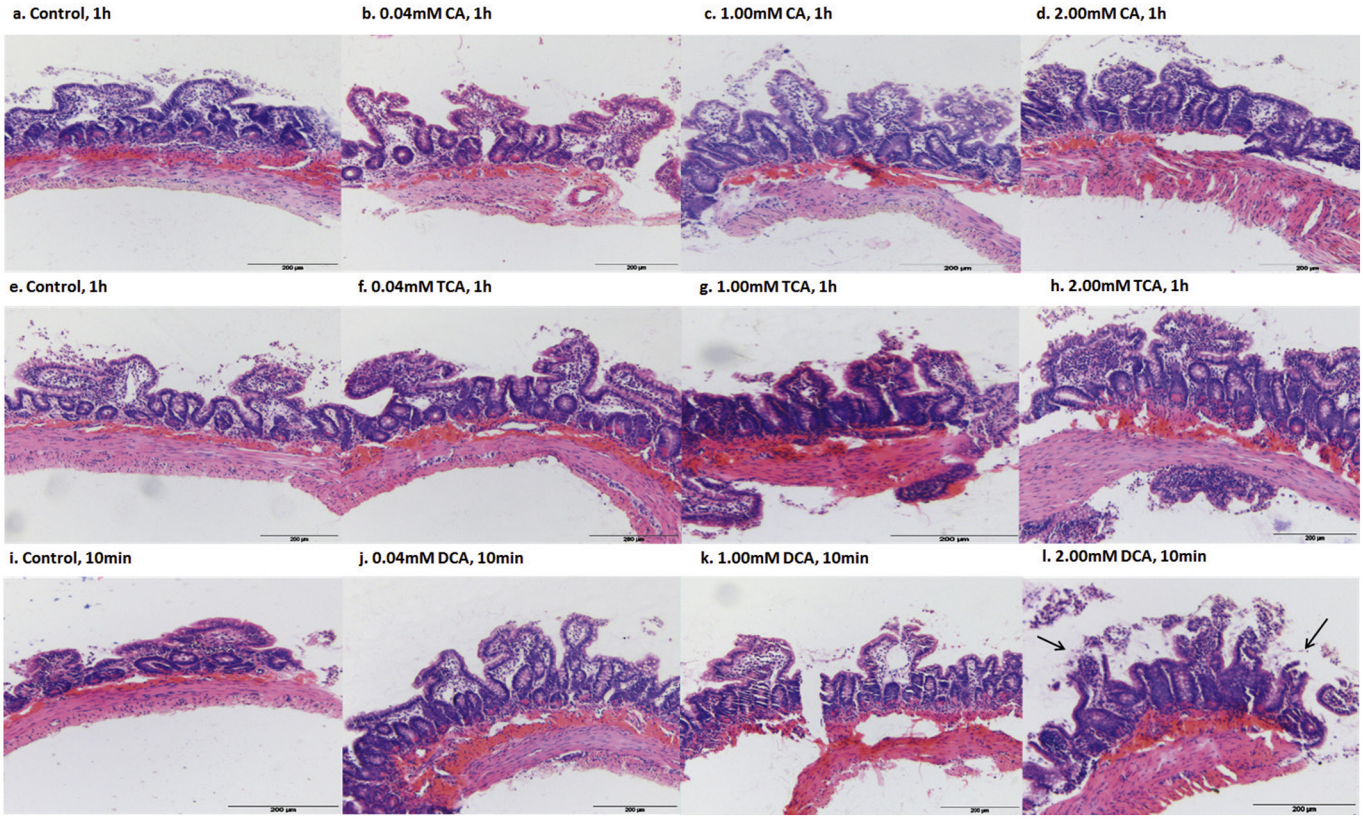

Fig. 2. Effect of bile acids on the morphology of rat PCIS.
Morphological evaluation of rat ileum PCIS incubated in the absence or presence of cholic acid (CA) (a–d), taurocholic acid (TCA) (e–h) and cholic acid (CA) (i–l), respectively at 0.04 (b, f, j), 1.00 (c, g, k) and 2.00 (d, h, l) mM. No obvious decrease in morphological integrity is seen for sections a–j In contrast sections k and l show flattening of the epithelial cells and discontinuous epithelial lining (indicated by arrows) indicating loss of viability of the enterocytes. Staining: hematoxylin and eosin. Scale bar is 200 µm.

PCIS are depicted in Fig. 4d and e, respectively. Passive diffusion was determined using the average values of taurocholic acid uptake at 37 °C from human jejunum and colon, as these values were shown to be the same at 37 °C and 4 °C in these regions in rat PCIS (see above, study on regional differences), indicating that also at 37 °C only passive diffusion takes place. Active uptake by human ASBT was shown to reach a plateau from 1.00 mM of taurocholic acid and the concentration-dependent uptake could be fitted according to the Michaelis-Menten equation (Fig. 4f). The affinity of the human ASBT for taurocholic acid is lower than that of rat ASBT (K_m of 0.27 and 0.14 mM, for human and rat respectively) but the capacity of taurocholic acid uptake is higher in human ileum (V_{max} in human ileum PCIS and rat ileum PCIS were 2.32 and 1.13 nmol/mg protein/min, respectively).

3.4. Inhibition of bile acid uptake by GSK20, fluvastatin and simvastatin

Fluvastatin inhibited the active uptake of taurocholic acid in rat ileum PCIS dose dependently, with 100% inhibition at 0.25 mM. Simvastatin partly inhibited the uptake of taurocholic acid to a maximum of 50% of the active uptake at 0.1 and 0.25 mM. Fluvastatin and simvastatin did not affect the passive uptake of taurocholic acid. GSK 20 appeared a potent inhibitor of the active uptake of taurocholic acid. It inhibited the active uptake completely at all concentrations, between 0.002 and 0.1 mM, as shown in Fig. 5. After 10 min incubation in the presence of GSK20, the slice total bile acid content was lower than that in the control, possibly due to inhibition of reuptake of excreted bile acids during the preincubation period (results not shown).

4. Discussion

In this study we investigated the applicability of rat and human PCIS as *ex vivo* models to study regional and species differences in bile acid uptake and ASBT-mediated transport in the rat and human intestine. The transport of three different bile acids, *i.e.* CA, TCA, DCA, was measured in this study at 4 and 37 °C in parallel.

The toxicity of ASBT substrates and bile acid uptake in different regions, *i.e.* duodenum, jejunum, ileum and colon, of the rat intestine and jejunum, ileum and colon of the human intestine was assessed first. It was shown that only deoxycholic acid was toxic at concentrations above 1 mM, and we selected 40 µM as concentration for further studies for all three bile acids. The toxicity of DCA was reported before and is well-known (Palmeira and Rolo, 2004; Petruzzelli et al., 2007; Perez and Britz, 2009; Barrasa et al., 2013).

At 4 °C only passive diffusion of the three bile acids can occur, which resulted in a linear increase of the uptake rate with increasing medium concentration of the bile acids. Passive diffusion of deoxycholic acid was higher than that of cholic acid and taurocholic acid. This was expected based on the different number of hydroxyl groups between the three bile acids which is inversely related to the rate of diffusion (Dietschy, 1968). Both cholic acid and taurocholic acid have three hydroxyl groups, which make them more hydrophilic and less permeable to the cell membrane. However, a drawback of this method of assessing passive diffusion is that the altered membrane fluidity and molecule movement at 4 °C can influence passive diffusion (Webborn et al., 2007; Lave et al., 2008; Zamek-Gliszczynski et al., 2013). However this influence is generally considered to be limited. Interestingly in human jejunum and colon, the passive uptake at 4 °C was similar to the uptake at 37 °C. Since ASBT is not expressed in these regions and uptake

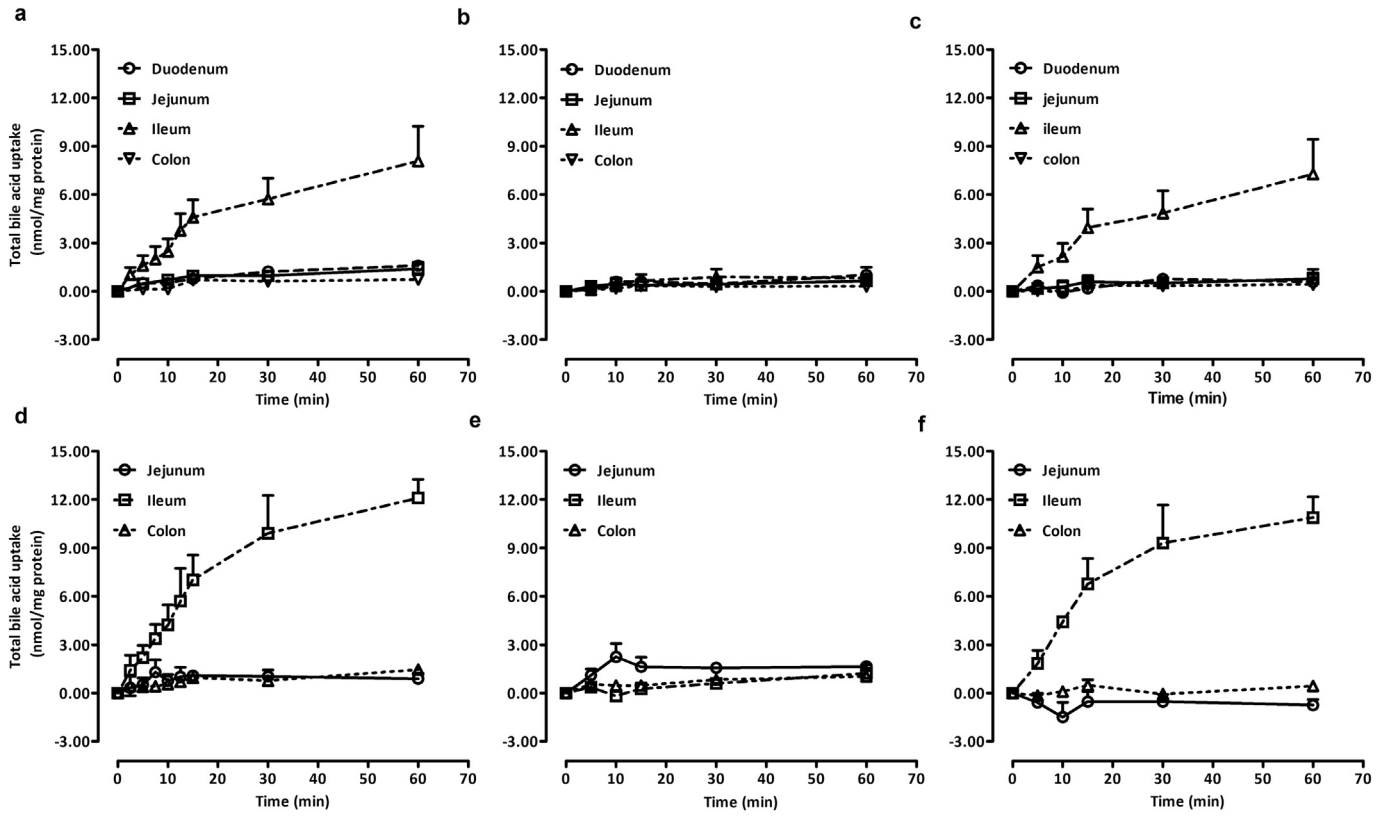

Fig. 3. Uptake of taurocholic acid in rat and human PCIS.
Time-course of taurocholic acid uptake in rat (a, b c) and human (d, e, f) PCIS incubated with 0.04 mM taurocholic acid. Panel a represents total uptake, *i.e.* passive and active uptake at 37 °C in rat duodenum (n = 4), jejunum (n = 6), ileum (n = 5) and colon (n = 4), while panel b shows the passive uptake at 4 °C in rat duodenum (n = 3), jejunum (n = 4), ileum (n = 4) and colon (n = 3). Active uptake in rat intestine is calculated by subtraction of passive uptake from total uptake and is depicted in panel c. Total uptake and passive uptake in the human intestine are depicted in d and e, respectively for jejunum (n = 4), ileum (n = 4), and colon (n = 3), and the resultant active uptake is shown in panel f. All values were corrected for the natural bile acid content in rat and human PCIS. Both in human and rat, the active uptake is present in the ileum only and is linear up to 15 min of incubation with taurocholic acid. Data are presented as mean ± SEM.

may be limited to passive transport only (Fig. 3), this indicates that the influence of altered membrane fluidity and molecule movement at lower temperature on the uptake of bile acids was indeed limited in PCIS. The higher uptake of each of the three bile acids at 37 °C in ileum PCIS than at 4 °C indicates active transporter-mediated uptake in these ileum slices.

Subsequently, we further characterized the transporter-mediated transport by measuring the concentration-dependent uptake of cholic acid, taurocholic acid and deoxycholic acid in rat PCLS. At concentrations higher than 1.00 mM uptake saturation was seen (Fig. 4e and c), indicating that the uptake of bile acids was mediated by active transport in rat and human ileum. We identified ASBT as the transporter responsible for this carrier-mediated uptake in rat PCIS as this uptake could be fully inhibited by the potent and selective ASBT inhibitor GSK20. In addition, fluvastatin and simvastatin, known to be inhibitors of ASBT, showed dose dependent inhibition of taurocholic acid uptake. To the best of our knowledge, this is the first report on using PCIS as *ex vivo* model to study the ASBT-mediated bile acid uptake and showing for the first time the functional characteristics of the human ASBT *ex vivo*.

ASBT-mediated transport was further characterized with respect to its kinetic parameters for three different bile acids in PCIS of the ileum. As expected, the uptake of taurocholic acid was high in rat and human ileum PCIS whereas little to no active uptake of taurocholic acid occurred in other regions (Fig. 3c and f), in concordance with the exclusive localization of ASBT in the ileum (Shneider et al., 1995; Alrefai and Gill, 2007; Lefebvre et al., 2009). This indicates that in these regions passive diffusion of bile acid is the main uptake pathway(Krag and Phillips, 1974; Dawson, 2011). Besides ASBT, OATP's were investigated as potential apical bile acid transporter *in vivo* and *in vitro*. Previous studies investigating expression of the members of OATP family have shown that both human OATP1A2 and rat Oatp1a5 are expressed at low levels on the apical membrane of the jejunum but that they are probably of minor importance for bile acid uptake (Shneider, 2001; Dawson et al., 2009). This is now confirmed by our data, as we found no active uptake of bile acids in slices of the rat and human jejunum where ASBT is not expressed. Moreover, the ASBT specific inhibitor GSK20 completely inhibited TCA uptake in rat ileum PCIS.

One of the important characteristics of PCIS is that all transporters are present in their physiological level and localization, including transporters possibly involved in bile acids handling by the intestine, including OATP, MRP1 and 3, BABP and OST α/β. The influence of OATP on bile acid uptake in the ileum slices is probably very limited in our study, as GSK20 completely inhibited the active uptake. The MRPs, bile acid binding protein and OST α/β will influence the final accumulation in the slices but do not influence the initial uptake rates and therefore do not interfere with our conclusions on ASBT function.

In Table 2 the apparent Km and apparent Vmax for bile acid uptake are presented. However, based on the conclusions presented above that the uptake is most likely exclusively ASBT-mediated, and taking into account that the Km value refers to the extracellular bile acid concentration, we believe that these values represent the physiological values for the Km and Vmax for ASBT. The rank order we found for the affinity of ASBT for bile acids (Table 2) is also seen in other studies where the highest affinity was found for conjugated bile acids (Schiff et al., 1972; Northfield and McColl, 1973; Craddock et al., 1998;

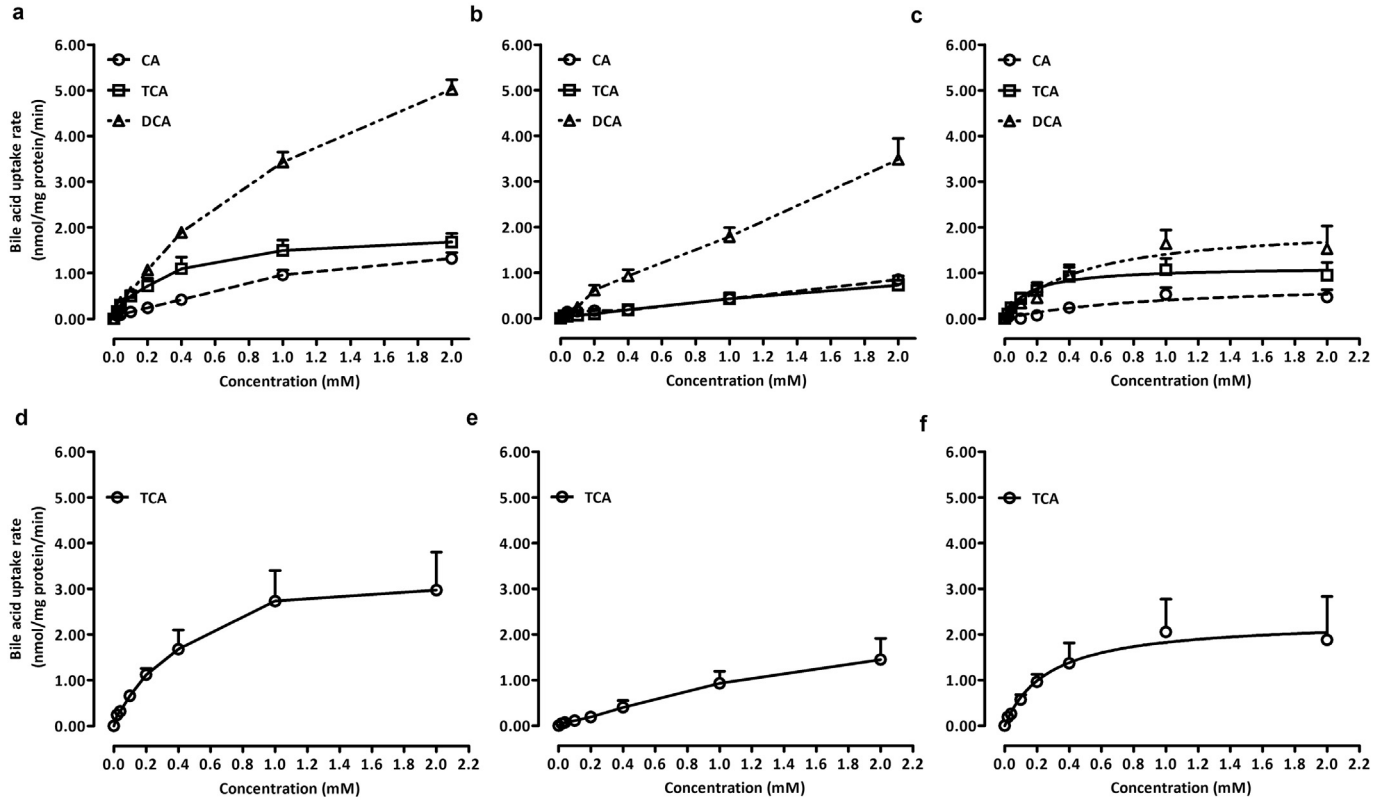

Fig. 4. Concentration-dependent uptake of bile acids in rat and human ileum.
Rat PCIS were incubated with cholic acid, deoxycholic acid and taurocholic acid (up to a concentration of 2.00 mM) for 10 min, while only taurocholic acid was used for human PCIS. Panel a represents the total uptake, *i.e.* passive and active uptake at 37 °C of cholic acid (CA) (n = 6), taurocholic acid (TCA) (n = 4) and deoxycholic acid (DCA) (n = 3) in rat ileum and panel b shows the passive uptake at 4 °C of cholic acid (n = 6), taurocholic acid (n = 3) and deoxycholic acid (n = 3). Active uptake of the three bile acids was calculated by subtraction of the passive uptake from the total uptake and is depicted in c. Total uptake (n = 4) and passive uptake (see the note below) of taurocholic acid by human ileum PCIS is depicted in d and e, respectively. Active uptake in human ileum is shown in panel f. All values were corrected for the natural bile acid content of rat and human PCIS. Data are presented as mean ± SEM. The active uptake in c and f was fitted to the Michaelis-Menten equation. Note: Since passive diffusion was not measured in human intestinal slices, due to an insufficient amount of slices, the values of passive diffusion in panel e was estimated by taking the average values from human jejunum and colon (n = 3 and 4, respectively) at 37 °C, where no active uptake was found (see Fig. 3).

Table 2
Kinetic parameters of active uptake by ASBT.

	Bile acid	$K_{m,app}$ [a]	$V_{max,app}$ [a]
		[mM]	[nmol/mg protein/min]
Rat	Cholic acid	0.98 ± 0.69	0.80 ± 0.26
	Taurocholic acid	0.14 ± 0.03	1.13 ± 0.07
	Deoxycholic acid	0.47 ± 0.14	2.07 ± 0.24
Human	Taurocholic acid	0.27 ± 0.05	2.32 ± 0.15

[a] Active transport parameters of ASBT are presented for the three bile acids in mean ± SEM of 3 experiments.

Martínez-Augustin and de Medina, 2008). Discrepancies exist between studies regarding the affinity of ASBT for unconjugated bile acids in relation to the number of hydroxyl groups. Some studies indicate that the affinity is higher for dihydroxy bile acids than trihydroxy bile acids, while others found the opposite (Schiff et al., 1972; Balakrishnan and Polli, 2006; Balakrishnan et al., 2006). Based on the most recent review the affinity of ASBT for bile acids increases after glycine or taurine conjugation and in presence of fewer hydroxyl groups (Dawson, 2011), which is in line with our findings regarding the rank order in affinity of ASBT being taurocholic acid > deoxycholic acid > cholic acid.

Our results also showed a different V_{max} of rat ASBT for the three bile acids. A possible explanation regarding this difference in V_{max} is

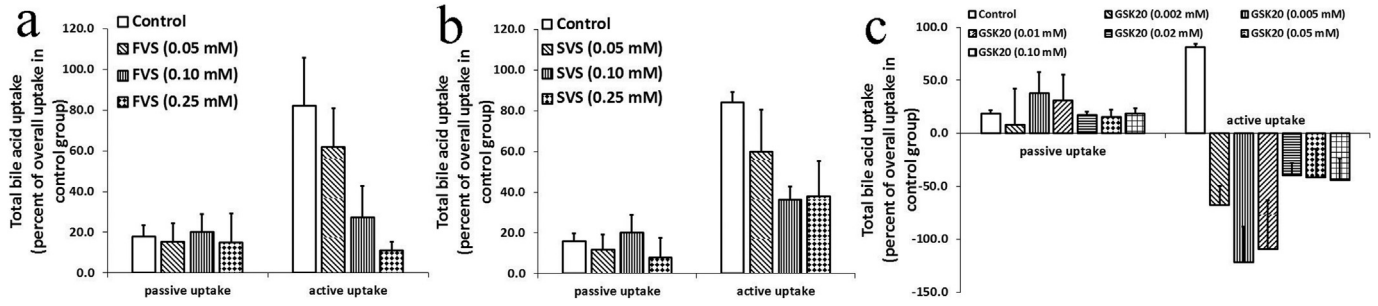

Fig. 5. Inhibition of uptake of taurocholic acid by simvastatin (SVS), fluvastatin (FVS) and GSK20.
The uptake of taurocholic acid was measured in rat ileum PCIS at 37 °C and 4 °C in the presence and absence of 0.05–0.25 mM simvastatin (a), 0.05–0.25 mM fluvastatin (b) and 0.002–0.1 mM GSK20 (c). Data are presented as % of total uptake in the control group and as mean ± SEM.

that bile acid binding to ASBT is a rate-limiting step which is faster when the affinity is higher (Balakrishnan et al., 2006). It should be noted that the V_{max} for deoxycholic acid was determined at concentrations where deoxycholic acid was shown to significantly decrease the viability of rat PCIS (Fig. 1 and Fig. 2 section l). Deoxycholic acid is known as a surface-active bile acid that can impair cell membranes and induce reactive oxygen species formation which eventually can lead to triggering of apoptosis or necrosis (Palmeira and Rolo, 2004; Petruzzelli et al., 2007; Perez and Britz, 2009; Barrasa et al., 2013). Because of the toxicity of deoxycholic acid at 2.00 mM the assessment of the V_{max} of ASBT for deoxycholic acid may not be accurate. However, uptake of deoxycholic acid was larger compared to the uptake of taurocholic acid and cholic acid also at the concentrations lower than 2.00 mM (Fig. 4), supporting the rank order of the V_{max} being deoxycholic acid > taurocholic acid > cholic acid. The values found in our study for Vmax (113 nmol/min/g tissue for taurocholate and 207 nmol/min/g tissue for deoxycholic acid) are well in the range of the values found in the literature for *in vivo* studies with isolated perfused loop experiments. Chen et al. (Chen et al., 2006) measured the uptake of a synthetic bile acid cholylsarcosine in a perfused loop of the ileum *in vivo* in Sprague Dawley rats. Based on their data a Vmax of 240 nmol/min/g tissue can be calculated. Moreover, Lewis and Root (1990) using a slightly different technique, found a Vmax of 655 nmol/min/g tissue for taurocholic acid also in Sprague Dawley rats. Taking into account the different strains of rats used, these data indicate that the Vmax found in our *ex vivo* model of PCIS reflect the *in vivo* activity of ASBT.

We found that the capacity of ASBT for taurocholic acid is higher in human PCIS than in rat ASBT. This may be explained by the higher abundance of ASBT in the human ileum that is required to reabsorb the much larger amounts of bile acids produced in human compared to rat. In previous studies the same trend is found for higher V_{max} values for the human ASBT paralog Na^+-taurocholate *co*-transporting polypeptide (NTCP), indicating species differences in the expression of bile acid uptake transporters or bile acid binding as discussed previously (Leslie et al., 2007; Hofmann and Hagey, 2008; Dong et al., 2013). However, the K_m of human ASBT for taurocholic acid is higher compared to rat ASBT.

5. Conclusions

In conclusion, this study shows that PCIS are a simple, reliable and fast *ex vivo* model to study passive uptake and ASBT-mediated transport of bile acids in the intestine of both rat and human. Also the toxicity of bile acids can be assessed with PCIS. ASBT activity was exclusively found in the ileum of both human and rat and was confirmed by inhibition by specific inhibitors. The activity of ASBT-mediated bile acid uptake was characterized on *ex vivo* level, and the K_m and V_{max} were assessed for cholic acid, taurocholic acid and deoxycholic acid. It was shown that in the rat ileum the conjugated bile acid taurocholic acid had the highest affinity for ASBT, while the capacity of ASBT is the highest for the unconjugated deoxycholic acid. Meanwhile the difference of ASBT activity between rat and human was evaluated, showing a higher activity for taurocholic acid in human. In the future PCIS can be applied to identify substrates and inhibitors of ASBT and to predict the extent of ASBT-mediated uptake in human PCIS.

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