Hepatic Transport Mechanisms of Cholyl-\text{-}\text{-}L\text{-}Lysyl\text{-}Fluorescein^{[\text{S}]}

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ABSTRACT

Cholyl-L-lysyl-fluorescein (CLF) is a fluorescent bile salt derivative that is being developed as an agent for determining in vivo liver function. However, the mechanisms of uptake and excretion by hepatocytes have not been rigorously studied. We have directly assessed the transport capacity of various hepatobiliary transporters for CLF. Uptake experiments were performed in Chinese hamster ovary cells transfected with human NTCP, OATP1B1, OATP1B3, and OATP2B1. Conversely, excretory systems were tested with plasma membrane vesicles from 

Sf21 insect cells expressing human ABCB11, ABCC2, ABCC3, and ABCG2. In addition, plasma clearance and biliary excretion of CLF were examined in wild-type, Abcc2\text{(-/-)}, and Abcc3\text{(-/-)} mice. Human Na\textsuperscript{+}\text{-}dependent taurocholate\text{-}cotransporting polypeptide (NTCP) and ATP\text{-}binding cassette B11 (ABCB11) were incapable of transporting CLF. In contrast, high\text{-}affinity transport of CLF was observed for organic anion\text{-}transporting polypeptide 1B3 (OATP1B3), ABCG2, and ABCC3 with \( K_m \) values of 4.6 ± 2.7, 3.3 ± 2.0, and 3.7 ± 1.0 \( \mu \)M, respectively. In Abcc2\text{(-/-)} mice biliary excretion of CLF was strongly reduced compared with wild\text{-}type mice. This resulted in a much higher hepatic retention of CLF in Abcc2\text{(-/-)} versus wild\text{-}type mice: 64 versus 1\% of the administered dose (2 h after administration). In mice intestinal uptake of CLF was negligible compared with that of taurocholate. Our conclusion is that human NTCP and ABCB11 are incapable of transporting CLF, whereas OATP1B3 and ABCG2/Abcc2 most likely mediate hepatic uptake and biliary excretion of CLF, respectively. CLF can be transported back into the blood by ABCG3. Enterohepatic circulation of CLF is minimal. This renders CLF suitable as an agent for assessing in vivo liver function.

Drug resistance\text{-}associated protein 2 (MRP2/Mrp2) (ABCC2/Abcc2) can transport bile salt metabolites (Akita et al., 2001). In various forms of intrahepatic cholestasis the biliary excretion capacity of ABCB11 and ABCG2 is decreased (Paumgartner, 2006). As a consequence, hepatic bile salt levels rise intracellularly, and, subsequently, the uptake transporters NTCP, OATP1B1, and OATP1B3 are down\text{-}regulated (Keitel et al., 2005). In primary biliary cirrhosis expression of bile salt uptake systems is reduced, whereas the canalicul transport systems BSEP and MRP2 are minimally affected and the basolateral salvage system is induced (Zollner et al., 2003, 2007). Hence, determination of the clearance of substrates of hepatocellular transport systems could be used as a measure for in vivo liver function. In the past, sulfobromophthalein and indocyanine green were used to measure liver function as a test for measuring transport activities, but the former was abandoned because of the possibility of severe systemic reactions, and the latter test is of concern for patients with iodine allergy (Sakka, 2007).
Cholyl-L-lysyl-fluorescein (CLF) is a fluorescent bile salt and has been introduced as a potential agent for assessing in vivo liver function (Milkiewicz et al., 2000). However, the transport systems involved in hepatic handling of CLF have not been investigated in molecular detail so far. Indications of the involvement of bile salt transporters in hepatic handling of CLF were found by several investigators. Mills et al. (1991) showed that the kinetics of CLF excretion into bile of rats is similar to that of glycocholate. In another study, Baxter et al. (1995) showed that CLF could induce phospholipid and cholesterol output similar to what was found for glycocholate. Because ABCC2/Abcc2 is also capable of mediating the transport of (modified) bile salts this transporter should also be considered as a potential candidate for biliary CLF excretion. However, Mills et al. (1999) concluded from a study with normal and TR(−) (Abcc2-deficient) Wistar rats that Abcc2 was hardly involved in the biliary excretion of CLF, based on the observation of similar biliary excretion in both strains. Carboxyfluorescein is an anion and a substrate for ABCG2 (Lee and Piquette-Miller, 2001). In this respect the fluorescence moiety of CLF could render CLF as an ABCG2/Abcg2 substrate. An indirect indication that CLF could be a substrate of ABCG2 was seen by Maglova et al. (1995). The excretion from couplets of cholylglycylamidofluorescein (CGamF), which resembles CLF, could partially be inhibited by dinitrophenyl-glutathione, a model substrate for ABCG2 (Evers et al., 1998). CLF is going to be introduced as a novel agent for determination of in vivo liver function in a clinical setting. It is therefore crucial to define the mechanisms of uptake and excretion by the hepatocyte. Altered plasma clearance in patients with liver disease will reflect a disturbance in the transport systems involved in hepatic handling of CLF. Because different transporters are regulated via different mechanisms, plasma clearance studies in patients can only be properly interpreted if it is known which transporters are involved. We made the surprising finding that the established human bile salt transporters NTCP and ABCB11 (BSEP) are not at all capable of transporting CLF. Instead transport is mediated via OATP1B3 and ABCC2.

Materials and Methods

Materials. CLF was a generous gift from Norgine International Ltd. (Harefield, UK). [3H]Glucose (52 Ci/mmol) and [3H]Tyrosine (1.19 Ci/mmol) were obtained from PerkinElmer Life and Analytical Sciences (Waltham, MA). [3H]Dextran-175-D-glucuronide (40.5 Ci/mmol) was purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). [3H]2,4-Dinitrophenyl-S-glutathione (DNP-SG) synthesis was performed as described previously (de Waart et al., 2006). Cellulose-acetate membrane filters were bought from Whatman Schleicher and Schuell (Dassel, Germany). Creatine-phosphate was purchased from Roche Diagnostics (Almere, The Netherlands). Creatine-kinase was bought from Roche Diagnostics (Mannheim, Germany). All other chemicals and reagents were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands).

Animals. Abcc2(−/−) mice were bred at the National Cancer Institute (Amsterdam, The Netherlands). The production and characterization of Abcc2(−/−) and Abcc3(−/−) mice have been described by Vlaming et al. (2006) and Zelcer et al. (2006), respectively. All mice were bred against a FVB background.

Generation of CHO Cells Stably Expressing NTCP and Cell Lines. cDNA for human NTCP (Hagenbuch and Meier, 1994) was cut with EcoRV and HindIII, and the coding region was ligated into pcDNA3/FRT (Invitrogen, Carlsbad, CA). CHO FlpIn cells (Invitrogen) were transfected with the resulting construct by using Lipofectamine 2000 (Invitrogen). Stably transfected cells were selected with 550 µg of hygromycin B in HAM-F12 medium (Invitrogen). Transfected cells were cloned with the limiting dilution method. Clones expressing functional NTCP were identified by transport assays with radioactively labeled taurocholate (TC) (see below). To further characterize the cloned cells, immunofluorescence localization of NTCP using a polyclonal antibody against NTCP (Kunz-Ublck et al., 1997) was performed as described previously (Huber et al., 2007). CHO cells expressing OATP1B1, OATP1B3, and OATP2B1 have been described previously (Treiber et al., 2007; Gui et al., 2008).

Transport Experiments with Stably Transfected Cell Lines. For all transport experiments, cells grown on 3-cm culture dishes were cultured for 24 h in media supplemented with 5 mM sodium butyrate to increase expression levels of transfected transporters (Palermo et al., 1991). Functionality of NTCP expressing CHO cells was assessed by transport studies with radioactively labeled taurocholate as described by Huber et al. (2007). Transport experiments with CLF were performed in the same buffers as for the radioactive substrates above. For visualization of CLF uptake, cells were inspected immediately after the uptake experiment with a DM-IRBE inverted microscope (Leica, Wetzlar, Germany) equipped with a ORKA-ER camera (Hamamatsu Photonics, Hamamatsu City, Japan). To determine uptake of CLF, cells were solubilized by the addition of 2 ml of 1% (w/v) Trition X-100. After complete solubilization, 1.5 ml of the solution was used to measure fluorescence in a PerkinElmer Life and Analytical Sciences LS-5 luminescence-spectrometer set at λex 486 nm (slit 10 nm) and λem 520 nm (slit 5 nm). Protein was determined with the bicinchoninic acid method and a kit from Interchim (Montluçon, France). Transport data from OATP-expressing cell lines were corrected for binding by subtracting 0-min time points (blank values) and, except for data in Fig. 2, B and C, data were normalized per minute. Kinetic analysis was performed with nonlinear regression of the data to the Michaelis-Menten equation by using Prism version 4.00 (GraphPad Software Inc., San Diego, CA).

Preparation of Membrane Vesicles. ABCC3 and ABCB12 recombinant baculovirus were a kind gift from Prof. P. Borst (Division of Molecular Biology, The Netherlands Cancer Institute, Amsterdam, The Netherlands) (Zelcer et al., 2001; Breweld et al., 2004). ABCB11 recombinant baculovirus was a kind gift from Dr. R. Thompson (Division of Gene and Cell Based Therapy, King’s College London School of Medicine, London, UK) (Byrne et al., 2002). The production of ABCB11, ABCB2, ABCC3, and ABCG2 recombinant baculovirus was as described previously (Byrne et al., 2002; de Waart et al., 2006, 2009). S2/S2 cells grown at 27°C were infected with ABCB11, ABCB2, ABCC3, and ABCG2 cDNA-containing baculovirus. Cells were harvested at 2 to 4 days after infection. Membrane vesicle preparation was as described previously (de Waart et al., 2009).

Western Blotting and Protein Analysis. Membrane vesicles were fractionated by 6% SDS-polyacrylamide gel electrophoresis and blotted on nitrocellulose membranes (Whatman Schleicher and Schuell), which were blocked in phosphate-buffered saline/5% milk powder/0.05% Tween 20. The following antibodies were used: anti-his probe, sc-803 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); anti-ABCG2, BXP-21 (Maliepaard et al., 2001); anti-ABCC2, M116 (Scheffer et al., 2000); and anti-ABC3, M1121 (Scheffer et al., 2000). Immune complexes were visualized with horseradish peroxi-dase-conjugated immunoglobulins and detected by using chemiluminescence (GE Healthcare, Little Chalfont, Buckinghamshire, UK).

Transport Assays with Plasma Membrane Vesicles. Transport studies with membrane vesicles were performed by using the rapid filtration technique as described previously (de Waart et al., 2009). Radiolabel was measured with a scintillation counter. When CLF was used as a probe, filters were placed in a glass tube, 0.1%
Triton X-100 was added, and the tubes were vortexed. Samples were pipetted in 96-well plates (Kartell, Noviglio, Italy), and the amount of CLF was quantitated by measuring fluorescence at λ_{ex} (485 nm) and λ_{em} (520 nm) with a NOVOstar microplate reader (BMG-Labtech, Offenburg, Germany).

**Animal Experiments.** Male mice were housed in a pathogen-free animal facility on a 12-h light/dark cycle. All animal experiments were approved by the institutional animal care and use committee of the Academic Medical Center. Mice were anesthetized with a combination of Hypnorm (VetaPharma, Leeds, U.K.; 11.8 mg/kg fentanyl and 0.37 mg/kg fentanyl-citrate) and diazepam (Centrafarm, Etten-Leur, The Netherlands; 5.9 mg/kg valium). Body temperature was maintained at 36 ± 1°C on thermostated heating pads. For clearance studies, mice were infused with CLF by injecting 100 μl (2 mM) of CLF into the tail vein. Subsequently, blood was drawn from the carotis at the indicated time points. Blood samples were deproteinated by the addition of two volumes of methanol, and the amount of CLF in the supernatant was quantitated by measuring fluorescence as described above.

For biliary secretion studies, gallbladders were cannulated with PE10 polyethylene tubing, and 100 μl (1 mM) of CLF was injected into the tail vein. Bile was collected in 10-min fractions; liver and blood were harvested at the end of the experiment. Homogenized livers were deproteinated by adding two volumes of MeOH, and bile and blood samples were diluted with 0.1% Triton X-100. The amount of CLF was quantitated by measuring fluorescence as described above.

For intestinal uptake studies of TC and CLF, mice were anesthetized, and the gallbladder was cannulated as described above. Mice received both TC and CLF by injecting 100 μl (2 mM) of CLF into the ileum. Bile was collected every 15 min. Radioactivity was measured in a scintillation counter, and CLF was quantitated as described above.

**Results**

First, the role of different hepatocellular bile salt transporters in the hepatic uptake of CLF was investigated. For this purpose CHO cells stably expressing NTCP were generated (CHO-NTCP) and characterized. Figure 1A shows no expression of NTCP in wild-type CHO cells but clear plasma membrane expression of NTCP in stably transfected cells. Furthermore, uptake of TC into these cells was strictly sodium-dependent and saturable with increasing taurocholate concentrations (Fig. 1B). The K_m and the V_{max} values were 16.0 μM and 6738 pmol TC/mg protein/min, respectively, for sodium-dependent transport of TC (Fig. 1B). No sodium-dependent uptake of CLF mediated by NTCP could be observed in these cells as detected by fluorescence microscopy (Fig. 2A) and quantitative fluorimetry of CLF (Fig. 2B). Uptake of CLF into NTCP-expressing cells was negligible, sodium-independent, and comparable with transport in wild-type cells (Fig. 2B). Next, uptake of CLF mediated by OATP1B1, OATP1B3, and OATP2B1 was examined in wild-type and stably transfected CHO cells. High time-dependent transport rates of CLF were seen only in OATP1B3-expressing CHO cells (Fig. 2, A and C). We consistently observed low, but significant, uptake of CLF by OATP1B1 cells, but not by OATP2B1 cells (Fig. 2, A and C), indicating that CLF is not a substrate for OATP2B1. OATP1B3-mediated transport was concentration-dependent (Fig. 2D) with K_m and V_{max} values of 4.6 ± 2.7 μM and 213 ± 42 pmol CLF/mg protein/min, respectively (mean of three independent determinations). In the case of OATP1B1, uptake experi-
higher than in control vesicles (Fig. 4, C and D, respectively). However, ABCC2- and ABCC3-containing membrane vesicles showed much higher CLF transport rates compared with control wild-type S/21 membrane vesicles (Fig. 4, E and F, respectively). ABCC2- and ABCC3-mediated transport of CLF was concentration-dependent (Fig. 4, G and H, respectively). The $K_m$ values were $3.3 \pm 2.0$ and $3.7 \pm 1.0$ μM for ABCC2 and ABCC3, respectively, and $V_{max}$ values were $436 \pm 215$ and $188 \pm 55$ pmol CLF/mg protein/min, respectively (mean of three independent determinations). Another model substrate for ABCC2 is DNP-SG, and CLF should inhibit transport of this compound. Half-maximal inhibition of DNP-SG transport with CLF was achieved at approximately 1 μM (Fig. 5A). Although we did not find any indication that CLF is a substrate for ABCB11, it contains the cholyl moiety; therefore, it might be able to inhibit bile salt transport via ABCB11. Indeed, TC transport could be inhibited by CLF in a dose-dependent fashion, and the concentration at which half-maximal transport was observed was approximately 10 μM (Fig. 5B).

To investigate the role of Abcc2 and Abcc3 in plasma clearance of CLF, we examined CLF levels in plasma of wild-type, Abcc2(−/−), and Abcc3(−/−) mice after a single injection of

Fig. 2. Uptake studies into CHO cells expressing hepatocellular uptake transporters. A, fluorescence pictures after incubation of CHO transporter cells with 1 μM CLF for 5 min. Fluorescent pictures (right) are complemented with phase-contrast pictures (left). Representative pictures are shown of 12 different experiments. B, transport of CLF mediated by NTCP in the presence (filled bars) and absence of sodium (empty bars) is given for 1 and 5 min. Shown is one of two experiments performed. For each time point $n = 4$. Significance was tested by using two-sided Student’s $t$ test. No significant differences were seen at 1 and 5 min for sodium versus choline buffer, respectively. C, wild-type cells and cells expressing OATP1B1, OATP1B3, and OATP2B1 were incubated with 1 μM CLF for 1 min (empty bars) or 5 min (filled bars). Shown is one of two experiments performed. For each time point $n = 4$. Significance was tested by using two-sided Student’s $t$ test. *, $P < 0.05$ for OATP-expressing CHO cells versus wild-type CHO cells. D, kinetics of OATP1B3-mediated CLF transport. Cells were incubated with increasing concentrations of CLF for 45 s. Shown are uptake rates corrected for 0-s binding. Data in B–D represent means ± S.D. $K_m$ and $V_{max}$ values are the mean of three independent determinations.
TC was recovered (Fig. 8). Hence, uptake of CLF in the ileum of wild-type FVB mice. Thereafter, bile was collected, and the amounts of [3H]TC and CLF excreted in bile were measured. At 4.5 h after ileal injection of both compounds into the tail vein. Clearance of CLF was strongly impaired in Abcc2(−/−) mice in comparison with wild-type mice but it was not affected in Abcc3(−/−) mice (Fig. 6). To examine the role of Abcc2 in biliary output of CLF, infusion experiments were performed with wild-type and Abcc2-deficient mice. Biliary excretion of CLF was very much delayed in Abcc2(−/−) mice (Fig. 7A). As a consequence, almost 70% of the CLF dose was excreted into bile in wild-type mice within 20 min, whereas in the same time span less than 2% was excreted into bile in Abcc2(−/−) mice. At 120 min after administration (Fig. 7B), the cumulative biliary excretion of CLF was still significantly higher in wild-type mice than in Abcc2(−/−) mice (85 versus 32% of the administered dose), which resulted in a significantly higher hepatic retention of CLF in Abcc2(−/−) versus wild-type mice (64 versus 1% of the administered dose) and higher blood levels in Abcc2(−/−) versus wild-type mice (1306 ± 749 versus 83 ± 21 nM). All of these data indicate that in mice Abcc2 is the main transporter responsible for the biliary excretion of CLF. In line with the observations from Fig. 7A, we found that the biliary output of CLF was not affected in Abcc3(−/−) mice in comparison with wild-type mice, and we found no differences in plasma and liver CLF contents (Fig. 7B).

After excretion into bile and delivery into the duodenum, bile salts are taken up in the ileum via the apical sodium-dependent bile acid transporter (ASBT) (Wong et al., 1995). To investigate whether CLF is taken up in the intestine, equimolar amounts of CLF and [3H]TC were injected into ileum of wild-type FVB mice. Thereafter, bile was collected, and the amounts of [3H]TC and CLF excreted in bile were quantified. At 4.5 h after ileal injection of both compounds only 2% of the CLF was recovered in bile, whereas 68% of the [3H]TC was recovered (Fig. 8). Hence, uptake of CLF in the terminal ileum is minimal. Apparently, CLF is not a good substrate for the intestinal uptake system Asbt.

**Discussion**

In this article we show that uptake of CLF into hepatocytes is not likely to be mediated by NTCP, because we did not find any uptake of CLF into CHO cells expressing human NTCP. This was not because of a nonfunctional protein, because these cells were fully capable of mediating the uptake of the natural occurring bile salt TC. The ideal counterpart of hepatic NTCP is ASBT, and it mediates the transport of conjugated and unconjugated bile salts (Dawson et al., 2009). We obtained indirect evidence to suggest that CLF is not transported via (murine) Asbt. Whereas TC injected in the ileal lumen of wild-type mice was very efficiently recovered in bile, we found almost no CLF. These data suggest that both NTCP and Asbt, which are homologous sodium-dependent bile salt transporters, are unable to transport CLF. This result compares favorably with studies of bile salts conjugated via the side chain to fluorescein. These bile salts are very poorly absorbed in the small intestine of rats (Holzinger et al., 1997). In contrast to CLF, another bile salt conjugate, taurocholyl-chlorambucil, was found to be a substrate for human NTCP (Kullak-Ublick et al., 1997). The latter compound is a conjugate at the 3-OH group of the bile salt, whereas CLF is conjugated at the side chain. In this context it is interesting that Baringhaus et al. (1999) determined the pharmacophore dates for uptake into the hepatocyte, which fits with the broad substrate specificity of these transporters (Hagenbuch and Meier, 2004).

Our new data are completely in line with this model. The less specific bile salt transporters, OATP1B3 and, to a lesser extent, OATP1B1, turn out to be more likely candidates for uptake into the hepatocyte, which fits with the broad substrate specificity of these transporters (Hagenbuch and Meier, 2004).

Our data demonstrate, quite surprisingly but conclusively, that ABC2/Abcc2, not ABCB11, is the most prominent transporter responsible for biliary excretion of CLF. In mice, the large majority of CLF in plasma is excreted into bile via Abcc2. It may be argued that the substrate specificity of human ABC2 can be different from that in mice. However, in plasma membrane vesicle assays we demonstrated that transport of CLF via human ABCB11 is insignificant compared with that via human ABC2. Therefore, in vitro studies with CLF support the notion that the side chain of bile...
salts is essential for the substrate recognition by the bile salt transporters ASBT, NTCP, and ABCB11. It has been demonstrated for rat Abcb11 that unconjugated cholate is practically not transported (Gerloff et al., 1998). This is supported by the observation that in patients with a defect in bile acid conjugation practically no unconjugated bile acids are found in bile (Carlton et al., 2003). Early reports involving CLF suggested that ABCB11 was involved mainly in this process. First, Mills et al. (1991) showed that in rats the biliary excretion rate of CLF after jugular vein injection has similar kinetics as the excretion of glycocholate but different from that of fluorescein, which was slower. Therefore, the conclusion was drawn that CLF is also a substrate for ABCB11 like glycocholate. However, this experimental approach did not rule out the possibility that CLF is a substrate for ABCB2, but the transport characteristics resemble those of glycocholate via ABCB11. Other indirect evidence that CLF is transported via ABCB11 was provided by Baxter et al. (1995), who administered glycocholate and CLF to isolated perfused rat livers under recycling conditions and observed that CLF was capable of increasing the phospholipid and cholesterol output in a similar way as glycocholate. Again, this does not rule out the possibility that CLF is excreted via Abcc2 and, once in the canalicular lumen, is subsequently...
able on the basis of its detergent properties to accept phospholipids and cholesterol from the membrane. It was shown in the same study that rat liver has a much greater capacity to transfer glycocholate from perfusate to bile than CLF, and, concomitantly, the increase in phospholipid and cholesterol output was less with CLF in comparison with glycocholate (Baxter et al., 1995). All in all, no direct evidence has been provided to support the contention that CLF is (mainly) taken up by the hepatocyte via NTCP/Ntcp and/or excreted into bile via ABCB11/Abcb11. Our present data show decisively that CLF is not transported via these bile salt transporters. Opposite to this finding is that CGamF incontrovertibly is an ABCB11 substrate.

What can be the explanation? The substrates CGamF and CLF contain both a cholyl and fluorescein moiety, but the bridge between the two moieties differs. First, the bridge in CLF is increased in comparison with CGamF, 131 Da; therefore, the molecular mass of the molecule is larger. This could mean that substrates for ABCB11 have a size limit. The bigger the substrates become the lower the affinity for ABCB11. Furthermore, ABCB2 can possibly accommodate larger molecules than can ABCB11. Second, in general ABCC2 mediates transport of anions with one or,
Another way to identify the relevant transporters is the use of COOH groups in comparison with CGamF, which contains preferably, two negative charges. Because CLF contains two COOH groups in comparison with CGamF, which contains one, CLF might be changed into a good ABCC2 substrate. Another way to identify the relevant transporters is the use of knockout animals. Mills et al. (1999) used Abcc2-deficient, TR(−) rats and measured CLF in bile after injection of CLF into the jugular vein. The cumulative amount of CLF in bile was similar in TR(−) and normal Wistar rats after 30 min. Mills et al. (1999) concluded from these data that Abcc2 is not the main transporter for CLF. However, we now directly show that Abcc2 is the main canalicular transporter for CLF in mice. We performed a similar, but more extensive, study using wild-type and Abcc2(−/−) mice. In mice deficient in Abcc2, biliary excretion of CLF was strongly impaired and retained in the liver, which suggests that Abcc2 and not Abcb11 is the major transporter responsible for biliary excretion of CLF. Because there is still residual biliary transport of CLF in Abcc2(−/−) mice, a contribution of Abcb11 and/or Abcg2, albeit small, cannot be excluded. Furthermore, reinterpretation of the data from Mills et al. (1999) showing the biliary excretion (see Supplemental Fig. 1, the lower right panel) represents our recalculation of the excretion based on the cumulative data given in the other panels) presents a similar picture as in mice. Mills et al. show the cumulative excretion. If it is changed to excretion per minute the result is a peak for excretion of CLF in the Wistar rat, which is not seen for the TR(−) rat. The decrease in excretion of CLF in the Abcc2(−/−) mice is very similar as in the TR(−) rat.

As CLF is being developed as an agent for assessing liver function in patients it is crucial to know the exact mechanism of uptake into the hepatocyte and excretion into bile. Therefore, we examined human ABCB11- and ABCC2-mediated transport of CLF. No ABCB11-mediated transport was seen, whereas ABCC2 mediated high-affinity transport of CLF. This transport was time-dependent and saturable with a K_m value of 3.3 µM and V_max of 436 pmol CLF/mg protein/min. In light of a liver function test these observations are crucial, because ABCB11 and ABCC2 gene expression are differently regulated, which leads to a different sensitivity of their expression toward various pathological conditions. For example, although ABCB11 and ABCC2 protein levels and localization were found to be maintained in primary biliary cirrhosis stages I and II (Zollner et al., 2003, 2007), reduced protein levels and a disruption of canalicular localization have been reported for ABCC2 in advanced primary biliary cirrhosis (stages III and IV) (Kullak-Ublick et al., 2002; Kojima et al., 2003). In a pilot study CLF elimination was analyzed in liver cirrhosis patients and seemed to be impaired in comparison with healthy volunteers (Milkiewicz et al., 2000). Those cirrhotic patients suffered from high serum bilirubin and bile salt level, indicative of a cholestatic situation. In such conditions ABCC2 function may not only be impaired but ABCC3 expression may also be up-regulated (Zollner et al., 2003). A biopsy can determine whether patients have a cirrhotic liver. However, a less invasive test would be more favorable. CLF could be used as an agent for assessing in vivo liver function, because in our study ABCC2 and ABCC3 mediate the transport of CLF. Cirrhotic patients may have a lowered biliary excretion rate of CLF, but also a higher basolateral excretion caused by a higher expression of ABCC3. It is noteworthy that analysis of plasma CLF clearance would not be compromised by enterohepatic circulation, because ileal uptake was low in our animal study. In conclusion, our data are of importance for the interpretation of plasma CLF clearance in patients with liver disease.

References

Fig. 8. Intestinal uptake of TC and CLF. Appearance of TC and CLF in bile after ileal administration of 100-μl mixture of 2 mM TC and CLF in wild-type mice. Bile was collected after the indicated time points. Data represent means ± S.D. Cumulative biliary levels are expressed as the percentage of applied dose. Significance was tested by using two-sided Student’s t test. *, P < 0.05 for TC versus CLF uptake.

Fig. 9. Chemical structures of the fluorescent bile salt derivatives, CLF (top) and CGamF (bottom). In the chylol-derivatives R1 and R2 denote α-hydroxy groups.

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of the ideal and the hepatic Na+/taurocholate cotransporters of the rabbit. II. A reliable 3D QSAR pharmacophore model for the ideal Na+-taurocholate cotransporter. J. Lipid Res. 32:2283–2288.


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