MRP2 and the defence against drugs and toxins
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Chapter VI

Regulation of organic anion transporters in a new rat model of acute and chronic cholangitis resembling human primary sclerosing cholangitis
Regulation of organic anion transporters in a new rat model of acute and chronic cholangitis resembling human primary sclerosing cholangitis

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Background/Aims: Primary sclerosing cholangitis (PSC) is a cholestatic liver disease of unknown etiology. Although the primary defect affects cholangiocytes, cholestatic injury of hepatocytes may promote further liver damage. Since down-regulation of hepatocellular organic anion transporters is implicated in the molecular pathogenesis of cholestasis, expression of these transporters was determined in a novel rat model, which closely resembles human PSC.

Methods: Hepatic protein and mRNA expression of basolateral (Ntcp, Oatp1, 2 and 4) and canalicular (Mrp2, Bsep) organic anion transporters were analyzed 1, 4 and 12 weeks after induction of experimental PSC by 2,4,6-trinitrobenzenesulfonic acid (TNBS).

Results: Specific down-regulation of basolateral and canalicular transport systems except Oatp4 and Bsep proteins occurred during the acute phase of inflammation. In chronic cholangitis 12 weeks after TNBS Mrp2 protein and mRNA remained down-regulated by 40-50% of controls (P < 0.05). In addition Oatp1 protein was also reduced by 40 ± 13% (P < 0.05), whereas all other transporters returned to control values.

Conclusions: In chronic cholangitis only canalicular Mrp2 expression remained down-regulated. This might represent the first injury to hepatocytes in chronic cholangitis as an extension of liver injury from the level of cholangiocytes to hepatocytes in PSC.

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Keywords: Organic anion transporters; Acute and chronic cholangitis; Human primary sclerosing cholangitis

1. Introduction

Primary sclerosing cholangitis (PSC), a chronic cholestatic liver disease of unknown etiology, is characterized by chronic inflammation and destruction of intra- and extrahepatic bile ducts [1]. This process results in periductular fibrosis, which in turn causes the characteristic bile duct strictures seen on cholangiography [2]. Fibrosis and inflammation infiltrate the periportal parenchyma with further progression of the disease and lead to necrosis of periportal hepatocytes, formation of fibrous septa and finally cirrhosis [1]. To date, there is no effective treatment for this cholestatic liver disease. The goal of current symptomatic, anti-inflammatory and immunosuppressive therapy is to prevent further damage and destruction of bile ducts. However, the molecular pathomechanisms of cholestasis in acquired human cholestatic disorders, especially PSC and primary biliary cirrhosis (PBC), are largely unknown, but may disclose new specific therapeutic strategies in the future.

Studies of the pathophysiology of cholestasis and the molecular regulation of hepatocellular transport systems have been facilitated by the cloning and functional characterization of several hepatic organic anion transport systems [3]. Physiologically, organic anions are taken up from portal blood by a sodium-dependent taurocholate cotransporter (Ntcp/Slc10a1) and several members of a growing family...
of sodium-independent organic anion transporters (Oatpl/Sclc21a1, Oatp2/Sclc21a5, Oatp4/Sclc21a10). After rapid transhepatic transport to the canalicular membrane, organic anions are secreted into bile by two ATP-dependent export pumps, the bile salt export pump (Bsep/Abcb11) and the multidrug resistance protein 2 (Mrp2/Abcc2). Molecular studies in several animal models of cholestasis including bile duct ligation, estrogen and endotoxin treatment have shown a selective down-regulation of organic anion transporters at the basolateral and canalicular membrane [4–10]. In contrast to clinical entities like PSC and PBC, these animal models of cholestasis mainly represent human diseases for which treatment is well established.

In the two acquired chronic cholestatic disorders PSC and PBC, molecular pathomechanisms are largely unknown. Kulak-Ublick et al. [11] detected increased Oatp1 mRNA in four PSC patients, whereas another group found an abnormal expression of anion exchangers (AE2) in PBC patients [12,13]. In a recent study Zollner et al. [14] found no significant changes in organic anion transporter expression in liver biopsy samples of anicteric PBC patients (Ludwig stages I and II). The lack of systematic molecular studies on PSC is mainly due to the absence of a suitable animal model.

Recently, a novel rat model of chronic fibrosing cholangitis induced by local administration of the hapten reagent 2,4,6-trinitrobenzenesulfonic acid (TNBS) into the dilated bile duct has been developed and revealed the opportunity to study the pathomechanisms of cholestasis in an animal model resembling PSC [15]. In contrast to several previous animal models of experimental sclerosing cholangitis [16–21], this new model closely resembles the human disease entity of PSC with histologic, cholangiographic, serologic and immunologic similarities. The animals develop the classic diagnostic criteria of PSC with a cholestatic biochemical profile, anti-neutrophil cytoplasmic antibody (ANCA) production, irregularities of the bile ducts as shown in retrograde cholangiography, moderate fibrosis and mononuclear cell infiltrates of the portal tract predominantly consisting of macrophages and T-lymphocytes. The histology showed severe extrapancreatic cholangitis and bile duct proliferation with moderate portal infiltration 1 week after TNBS, but only minimal portal fibrosis. Whereas extrapancreatic cholangitis was almost resolved over the next 8 weeks, portal fibrosis progressed to a mild degree after 12 weeks [15].

In the present study, we have used this novel animal model to characterize the regulation of multiple hepatobiliary organic anion transporters in acute and chronic fibrosing cholangitis. Both basolateral and canalicular transport systems were down-regulated at the transcriptional and protein levels during the acute phase of inflammation, which finally led to a selective down-regulation of Ntcp, Oatp4 and Mrp2 mRNA levels as well as a reduction of Oatp1 and Mrp2 protein mass at the stage of chronic fibrosing cholangitis after 12 weeks.

2. Materials and methods

2.1. Animal models

Animal experiments were performed at the animal facility of Mainz University. As previously described [15], male and female inbred Lewis rats (body weight 175 g) were obtained from Harlan-Winkelmann, Borchen, Germany. Two weeks before induction of fibrosing cholangitis, the common bile duct was exposed after laparatomy and a mild stenosis was induced in each animal according to the method described by Rodriguez-Gary et al. [22]. Briefly, a double ligature was performed after placement of a removable small catheter (Implantofix, Braun, Melsungen, Germany) along the common bile duct to ensure a mild degree of stenosis. To induce cholangitis, the dilated common bile duct was exposed after relaparotomy and animals were injected with 50 mg/kg TNBS (Sigma, Deisenhofen, Germany) diluted in 10% (v/v) ethanol/0.9% (w/v) NaCl into the dilated common bile duct. Animals were then maintained on a 12 h day and 12 h night cycle with free access to standard chow and water. Livers were harvested on weeks 1, 4 and 12 after injection and immediately frozen in liquid nitrogen for protein and RNA analysis. Blood samples were obtained by cardiac puncture prior to removal of the livers. All study protocols were approved by the Federal Government's Animal Care Committee.

2.2. Northern analysis

RNA was isolated from whole liver by the Ultraspec™ phenol chloroform extraction procedure (Biotex Laboratories Inc., Houston, TX) according to the instruction manual, quantified spectrophotometrically at 260 nm and stored at −70 °C. Total RNA (10–20 µg) was denatured, electrophoresed on a 1% agarose/formaldehyde gel, transferred to a nylon membrane (Nytran 0.2; Schleicher&Schüll, Dassel, Germany) by overnight capillary blotting and UV-crosslinked (UV Stratalinker 1800, Stratagene, La Jolla, CA). Ethidium bromide staining of 18S and 28S bands was used to ensure equal loading for each sample. The membranes were prehybridized for 30 min at 60 °C in ExpressHyb™ solution (Clontech, Palo Alto, CA). After replacement with fresh ExpressHyb solution, hybridization was performed at 60 °C for 1 h after addition of specific rat complementary DNA (cDNA) probes labeled with [32P]dCTP (specific activity 106 cpm/µg) by a random primed method (High Prime®, Roehringer Mannheim, Mannheim, Germany). Blots were washed twice with 2 x SSC/0.05% SDS for 10 min at room temperature, followed by 2 x SSC/1% SDS for 20 min at 50 °C. Specific mRNA levels were detected after exposure of membranes to a Phosphorimager screen (Molecular Dynamics, Sunnyvale, CA) and quantified using a Phosphoimager and the ImageQuant™ software (Biorad). Specific and constitutively expressed probes used were as previously described [23]: Ntcp cDNA (0.9 kb EcoRI fragment), Oatp1 cDNA (0.73 kb PstI fragment), Oatp2 cDNA (3.7 kb EcoRV/BssHI fragment), Oatp4 cDNA (NcoI/BamHI fragment) [24], ectorAT-Pase cDNA (1.3 kb XbaI/PstI fragment) and GAPDH cDNA (1.25 kb PstI fragment). cDNA probes for Mrp2 [25] and Bsep were generated by polymerase chain reaction using rat liver cDNA as templates. Primers for a 0.58 kb Mrp2 fragment and a 0.65 kb Bsep fragment were used as previously described [23].

2.3. Western analysis

Preparation of liver microsomes and analysis of protein mass of various polypeptides were performed as previously described [26]. Protein concentrations were determined according to Bradford [27]. After determination of the linear range (25–200 µg), similar amounts of protein (75 µg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis [28], transferred to polyvinylidene fluoride blotting membranes (Biorad, Gelman Sciences, Roßdorf, Germany), and probed to either anti-Ntcp, fusion protein antiseraum [29], anti-Oatp1 fusion protein antiserum [6], anti-Oatp2 and anti-Oatp4 peptide antiseraun, anti-Mrp2 peptide antiseraun, monoclonal anti-Na+/K+-ATPase α-1 antibody (Upstate Biotechnology, 91
Lake Placid, NY) or anti-Bsep peptide antiserum [30]. Immune complexes were detected using horseradish peroxidase-conjugated donkey anti-rabbit IgG F(ab')2 fragments according to the ECL™ Western blotting kit (Amer-
controls, Little Chalfont, UK). Immunoreactive bands obtained by autoradiography were quantified by laser densitometry and analyzed by the ImageQuant™ software (Biorad).

2.4. Biochemical liver tests

Liver function tests including alkaline phosphatase (AP) and bilirubin as markers of cholestasis and aspartate aminotransferase (AST) and alanine aminotransferase (ALT) as markers of hepatocellular necrosis were determined by a standard autoanalyzer.

2.5. Statistical analysis

Statistical analysis between controls and TNBS-treated animals was performed using multivariate ANOVA with posttesting. Statistical significance was considered at \( P < 0.05 \). Data represent the mean ± SEM of at least four animals per group at each time point examined.

3. Results

3.1. Clinical chemistry

Induction of acute cholangitis in rats led to significant elevation of transaminases with maximum levels at week 1 (AST 504 ± 335 U/l, ALT 145 ± 125 U/l, \( P < 0.05 \), \( n = 4-5 \)) and hyperbilirubinemia (5.6 ± 6.6 mg/dl, \( P < 0.05 \)) compared to untreated controls (AST 44 ± 5 U/l, ALT 24 ± 3 U/l, bilirubin 0.03 ± 0.03 mg/dl) (Table 1). Liver function tests returned to normal values within the next 3 weeks, but increased again to a similar extent as in week 1 (AST 378 ± 159 U/l, ALT 105 ± 33 U/l) with chronification of the bile duct inflammation at 12 weeks. However, bilirubin remained at baseline levels indicating that no severe cholestasis occurred at this time point.

3.2. Steady-state mRNA levels

To determine whether mRNA expression of hepatocellular organic anion transporters was altered during acute and chronic inflammatory response to TNBS injection, we quantified steady-state mRNA levels of transport proteins exclusively localized either to the basolateral (Ntcp, Oatpl, 2 and 4) or the canalicular (Bsep, Mrp2) plasma membrane of the liver by Northern blots (Fig. 1). The most extensive down-regulation of all basolateral and canalicular transporters was observed 1 week after TNBS injection. All basolateral transporter mRNAs including Ntcp, Oatpl, 2 and 4 declined on average to 38 ± 20, 22 ± 9, 22 ± 9 and 22 ± 8% of control levels, respectively \( (P < 0.05, n = 4) \) (Fig. 2). While inflammatory bile duct lesions persisted, all basolateral transporters recovered to various extents, but Ntcp and Oatpl still remained significantly reduced at 12 weeks (Ntcp 66 ± 13%, Oatpl 56 ± 13%, \( P < 0.05 \)). In addition, mRNA levels of the canalicular export pumps Mrp2 and Bsep also declined initially to 19 ± 10 and 46 ± 16% of controls, respectively \( (P < 0.05, n = 4) \) (Fig. 3). Whereas the canalicular Bsep was fully recovered 12 weeks after TNBS, Mrp2 mRNA remained down-regulated to a similar extent as initially observed during the development of chronic fibrosing cholangitis up to 12 weeks (Fig. 3). In contrast to all transporters, mRNA levels of the housekeeping gene GAPDH remained unchanged at any time point after TNBS treatment.

Table 1
Clinical chemistry data of TNBS-treated rats

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>Week 1</th>
<th>Week 4</th>
<th>Week 12</th>
</tr>
</thead>
<tbody>
<tr>
<td>AST (U/l)</td>
<td>44 ± 5</td>
<td>504 ± 335</td>
<td>126 ± 15</td>
<td>378 ± 159</td>
</tr>
<tr>
<td>ALT (U/l)</td>
<td>24 ± 3</td>
<td>145 ± 125</td>
<td>77 ± 16</td>
<td>105 ± 33</td>
</tr>
<tr>
<td>AP (U/l)</td>
<td>294 ± 96</td>
<td>686 ± 234</td>
<td>441 ± 80</td>
<td>173 ± 36</td>
</tr>
<tr>
<td>Bilirubin (mg/dl)</td>
<td>0.03 ± 0.03</td>
<td>5.6 ± 6.6</td>
<td>0.05 ± 0.04</td>
<td>0.14 ± 0.04</td>
</tr>
</tbody>
</table>

* Liver function tests are given as means ± SD (\( n = 5 \)).
lateral and canalicular organic anion transporters also affect their protein expression, the protein mass of Ntcp, Oatp1, 2 and 4, Mrp2 and Bsep were quantitated by Western blot analysis (Fig. 4). Protein levels of Ntcp, Oatp1 and Oatp2 declined 1 week after TNBS treatment (23 ± 13, 22 ± 3 and 21 ± 7%, respectively, P < 0.05, n = 4), but Oatp4 protein mass remained largely unchanged. Later, all basolateral transporters recovered during the chronic stages of cholangitis except Oatp1, which was still slightly but significantly reduced by 40 ± 13% (P < 0.05, n = 5) (Fig. 5). Protein levels of canalicular membrane transporters Mrp2 and Bsep paralleled their mRNA levels. Mrp2 protein mass declined to 17 ± 18% 1 week after TNBS and remained down-regulated at 12 weeks (61 ± 28%, P < 0.05, n = 5). In contrast, Bsep slightly declined to 78 ± 9% (P < 0.05, n = 5) during acute cholangitis (1 week after TNBS) and rapidly returned to baseline within the next 3 weeks (Fig. 6). The decrease in organic anion transporter protein mass appeared to be specific since the basolateral anionic ion exchanger Na⁺, K⁺-ATPase remained unchanged (Fig. 4).

4. Discussion

The TNBS-induced animal model of fibrosing cholangiitis exhibits several characteristic features unique in human PSC including immunologic parameters (e.g. ANCA), cholangiographic appearance and histology. Bile duct proliferation and mononuclear infiltration of portal tracts initially predominate during the acute phase of inflammation (within 1 week after TNBS injection), whereas portal fibrosis and bile duct irregularities and strictures progressively develop within the next 3 months [15]. Since PSC is characterized by progressive cholestasis leading to secondary biliary cirrhosis, this animal model is particularly attractive to study the expression of a variety of hepatocellular organic anion transporters previously shown to be involved in the molecular pathogenesis of cholestasis [3]. Although the primary defect of PSC is at the levels of both small and large bile ducts, impairment of transporter expression might further promote end stage liver disease by the development of hepatocellular cholestasis with decreased elimination of hepatotoxic compounds.

In TNBS-induced cholangitis, acute inflammation occurred within 1 week after injection characterized by a 6–10-fold increase of transaminase levels and significant hyperbilirubinemia (Table 1). Although transiently recovered, both AST and ALT levels increased again by 4–7-fold of control values, when bile duct strictures developed at week 12. However, bilirubin remained normal indicating that TNBS-induced cholangitis resembles an earlier phase of human PSC when severe cholestasis is still absent (Table 1). These data are also in close agreement with previous observations in rats with TNBS-induced colitis, which do not develop any hyperbilirubinemia despite a concomitant cholestatic hepatobiliary disorder [31].

To characterize the effects of TNBS-induced cholangitis on the expression of hepatobiliary organic anion transporters known to play an important role in bile formation, steady-state mRNA and protein levels were determined at various time points during the development of chronic cholangitis. Steady-state mRNA levels of all hepatobiliary transporters including basolateral Ntcp and Oatps 1, 2 and 4 as well as canalicular Mrp2 and Bsep were significantly down-regulated by 40–80% compared to controls 1 week after TNBS treatment (Figs. 1–3). Later, most transporter mRNAs slowly returned to control values, except basolateral Ntcp and Oatp4 and canalicular Mrp2, which increased to approximately 50–60% of their controls during this period (Figs. 2 and 3). Changes in mRNA levels were not uniformly accompanied by similar alterations in protein levels (Figs. 4–6). Although Oatp4 mRNA declined and remained decreased for up to 12 weeks, its protein mass...
was unaltered at any time after TNBS application (Fig. 5). Although the cellular fate of Oatp4 protein is currently unknown, its protein turnover appears to be posttranslationally regulated in various forms of liver injury, since a similar observation of discordant Oatp4 expression of reduced mRNA and maintained protein was obtained in bile duct obstructed rats (Gartung et al., unpublished data). However, an Oatp4 specific difference in the sensitivity of mRNA and protein detection assays can not be ruled out. The discrepancy between mRNA and protein levels of Ntcp and Oatp1 remained within a 20% range and statistical significance might be explained by the various degrees of standard deviations in this in vivo model. The discrepancy between down-regulation of rat Oatp1 in TNBS-induced PSC and the previously described up-regulation of human OATP-A in human PSC may be explained by the fact that Oatp1 does not represent the rat homologue of the human OATP-A gene [32]. Nevertheless, the most consistent correlation was obtained for the canalicular Mrp2, which remained down-regulated to similar extents at both mRNA and protein levels at any time during TNBS-induced cholangitis (Figs. 4, 6).

<table>
<thead>
<tr>
<th>Protein</th>
<th>Control</th>
<th>1wk</th>
<th>4wk</th>
<th>12wk</th>
<th>MW (kDa)</th>
</tr>
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<tbody>
<tr>
<td>Ntcp</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>49.5</td>
</tr>
<tr>
<td>Oatp1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>80</td>
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<td>Oatp2</td>
<td></td>
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<td>80</td>
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<tr>
<td>Oatp4</td>
<td></td>
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<td></td>
<td>80</td>
</tr>
<tr>
<td>Mrp2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>190</td>
</tr>
<tr>
<td>Bsep</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>160</td>
</tr>
<tr>
<td>Na,K-ATPase</td>
<td></td>
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<td>80</td>
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</tbody>
</table>

Fig. 4. Effect of TNBS-induced cholangitis on protein mass. Membrane fractions were isolated from TNBS-treated and control rats \((n = 4-5)\), subjected to SDS-PAGE \((75 \mu g \text{ protein/lane})\), and subsequently blotted onto nylon membranes as described in Section 2. Representative immunoblots of two independent samples are shown for each organic anion transporter for up to 12 weeks after TNBS injection. Molecular weight \((MW)\) markers are given in kilodaltons.

![Graph of Protein Mass over Time](image)

Fig. 5. Densitometric analysis of basolateral transporter protein levels. The protein mass of various basolateral transporters shown in Fig. 4 was quantified by laser densitometry and expressed as a percentage of controls \((n = 4-5, \text{ mean } \pm \text{ SEM})\). Ntcp, Oatp1 and Oatp2 proteins were significantly down-regulated within 1 week after TNBS injection when compared to controls. In contrast to the recovery of Ntcp and Oatp2, Oatp1 protein still remained decreased by \(40 \pm 7\% \ (P < 0.05)\) at the stage of chronic cholangitis \((12 \ \text{ weeks after TNBS injection})\).

![Graph of Protein Mass over Time](image)

Fig. 6. Densitometric analysis of canalicular transporter protein levels. Whereas Bsep protein was only diminished during the acute phase after TNBS-induced cholangitis, Mrp2 protein mass was significantly down-regulated during both acute and chronic cholangitis \((P < 0.05)\). Data are given as the mean \(\pm \text{ SEM} \ (n = 4-5)\).
3 and 6). The normalized level of bilirubin 12 weeks after TNBS is in agreement with a recent report by Paulusma et al. [33] who found normal bilirubin levels even in the case of a 50% reduction in Mrp2 protein expression. However, excretion of other substrates of Mrp2 such as S-(2,4-dinitrophenyl)glutathione were reduced in correlation to the Mrp2 protein levels [33].

After injection of TNBS into the bile duct, acute extra- and intrahepatic cholangitis occurs, which results in activation of liver-derived mononuclear cells and macrophages with production of pro-inflammatory cytokines such as tumor necrosis factor alpha (TNFα) [15]. In terms of regulation, pro-inflammatory cytokines including TNFα and interleukin-1β (IL-1β) have recently been shown to regulate the transcriptional activity of the Ntcp and Mrp2 promoters in vitro [34]. Thus, induction of cytokines in TNBS-induced acute liver injury might be an important regulator of decreased organic anion transporter expression, although regulatory factors have only been identified for Ntcp and Mrp2, but are still lacking for Oatps and Bsep. However, similar to TNBS-induced liver injury, Bsep also remained more or less unchanged in endotoxin-induced cholestasis in contrast to the marked down-regulation of Mrp2 or Ntcp [35]. Since cytokines are still produced during chronic cholangitis albeit to a lesser extent [15], sustained down-regulation of the most susceptible transporter Mrp2 might be due to continuous production of regulatory cytokines. It is reasonable to speculate that sustained down-regulation of Mrp2 might extend liver injury to the level of the hepatocytes even after TNBS, although the primary defect occurs at the level of the cholangiocytes similar to human PSC. In contrast, down-regulation of Mrp2 might represent a protective mechanism by which the liver reduces further secretion of TNBS and other toxic metabolites into the bile ducts. In a model of TNBS-induced colitis, animals developed a concomitant cholestasis with retention of bile acids and reduced bile flow similar to the association of colitis and hepatobiliary disorders (e.g. PSC) in humans [31,36]. Immunofluorescence microscopy showed a marked decrease in Mrp2 protein expression after intracolonic TNBS treatment similar to previous observations in various cholestatic models induced either by endotoxin or biliary obstruction [5,31].

In summary TNBS-induced cholangitis represents the first suitable animal model of early stages of human PSC. The expression of transporters involved in bile formation is overall diminished during the acute phase of cholangitis, but in general returns to normal values within a short time. However, sustained down-regulation of Mrp2 up to 12 weeks after TNBS occurs and might represent either extension of liver injury from cholangiocytes to hepatocytes or a protective mechanism to diminish delivery of toxic compounds to the bile ducts. Although down-regulation of expression can also be observed for the basolateral Oatp1, its dysfunction should not be of any major physiological importance since other members of the Oatp family have a similar transport spectrum and may compensate for Oatp1 function. Thus, TNBS-induced cholangitis allows for the first time systematic expression studies of hepatobiliary transporters previously shown to be important players in the complex process of bile formation and cholestasis.

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