

Review

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The many facets of bile acids in the physiology and pathophysiology of the human liver

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Abstract: Bile acids perform vital functions in the human liver and are the essential component of bile. It is therefore not surprising that the biology of bile acids is extremely complex, regulated on different levels, and involves soluble and membrane receptors as well as transporters. Hereditary disorders of these proteins manifest in different pathophysiological processes that result in liver diseases of varying severity. In this review, we summarize our current knowledge of the physiology and pathophysiology of bile acids with an emphasis on recently established analytical approaches as well as the molecular mechanisms that underlie signaling and transport of bile acids. In this

review, we will focus on ABC transporters of the canalicular membrane and their associated diseases. As the G protein-coupled receptor, TGR5, receives increasing attention, we have included aspects of this receptor and its interaction with bile acids.

Keywords: bile salts; hereditary diseases; liver; membrane transport.

A general introduction to bile salts

Bile acids (BA) are essential for the absorption of dietary lipids and fat-soluble vitamins and also act as signaling molecules for diverse endocrine and paracrine functions (Keitel et al. 2008). Besides others, they can activate different BA receptors, such as the nuclear hormone farnesoid-X-receptor (FXR, NR1H4) (Makishima et al. 1999; Parks et al. 1999) and are ligands for G-protein coupled receptors such as the sphingosine-1 phosphate receptor 2 (S1PR2) (Studer et al. 2012) and the Takeda G protein-coupled receptor-5 (TGR5, Gpbar1) (Kawamata et al. 2003; Keitel et al. 2019b; Keitel et al. 2020) as well as the adhesion receptor $\alpha_5\beta_1$ integrin (Bonus et al. 2020; Gohlke et al. 2013). Synthesis of BA occurs in the liver through oxidation of cholesterol via two major pathways: the classic and alternative pathways (Figure 1). In the classic pathway, CYP7A1 represents the rate-limiting enzyme that catalyzes the conversion of cholesterol to 7 α -hydroxycholesterol, which leads after several additional enzymatic steps to the formation of the primary BA, namely cholic acid (CA) and chenodeoxycholic acid (CDCA) (Sarenac and Mikov 2018). Hydroxylation at position 27 of cholesterol through the first enzyme CYP27A1 in the alternative pathway leads to the formation of 27-hydroxycholesterol, followed by CYP7B1-mediated hydroxylation to produce CDCA finally. The amount of total BA from the alternative pathway under normal human physiological conditions is less than 10%. The up-regulation of the alternative pathway may occur when the main pathway is fully or partially disturbed. Both CA and CDCA undergo conjugation reactions in the liver

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with taurine and glycine (TCA, GCA, TCDCA, and GCDCA) on their side chains and with sulfate, glucuronic acid, and *N*-acetylglucosamine on the hydroxyl groups of the cholane scaffold. In bile, the relative proportions of bile acids excreted, e.g., as sulfates or glucuronides are less than 2% (Takikawa et al. 1985). The taurine- and glycine-conjugated BA are secreted into the gallbladder and finally excreted into the small intestine. Three biotransformation reactions may occur to the conjugated bile acids in the intestinal tract through bacterial enzymes (Ridlon et al. 2016). The deconjugation reactions of glycine and taurine, epimerization and dehydroxylation steps generate secondary BA such as deoxycholic acid (DCA) or lithocholic acid (LCA). More than 90% of the BA are re-absorbed across the gut epithelia and transported to the liver via specific transporters. The re-absorbed free BA again undergo conjugations with taurine and glycine. This process is called “the enterohepatic circulation of bile acids” (Hofmann 1984; Keitel et al. 2019a; Ridlon et al. 2006). The most common human BA are CA, CDCA, DCA, LCA, and UDCA (urso-deoxycholic acid), as well as their taurine- and glycine-conjugated forms. UDCA, which accounts for up to 4% of the human BA pool under physiological conditions (Lazaridis et al. 2001), is generated from CDCA through epimerization steps and used as a therapeutic agent, e.g., in cholestatic liver diseases such as primary biliary cholangitis (Paumgartner and Beuers 2002). While, UDCA treatment did not reduce adverse perinatal outcomes in women with intrahepatic cholestasis of pregnancy (Chappell et al. 2019), patients with heterozygous mutations in the phospholipid floppase ABCB4 (MDR3) treated with UDCA showed a mild disease course with transplant-free survival of 91% (de Vries et al. 2020). The hydrophilicity of the common free and conjugated BA decreases in the order UDCA > CA > CDCA > DCA > LCA, and taurine-conjugated > glycine-conjugated > free species. LCA represents the most lipophilic BA with toxic properties causing severe liver damage and biliary cholestasis. The use of animal models, especially genetically modified mice, plays an important role in studying the relationship between bile acid metabolism and human liver disease. In rodent livers, CDCA is hydroxylated at the 6 β -position by CYP2C70 to α -muricholic acid (α -MCA) and β -muricholic acid (β -MCA), representing further primary BA besides CA and CDCA (Takahashi et al. 2016). Microbial biotransformation including deconjugation, dehydroxylation, and epimerization leads to other secondary BA such as ω -MCA, MDCA (murideoxycholic acid), HCA (hyocholic acid), and HDCA (hyodeoxycholic acid) from α -MCA and β -MCA (Takahashi et al. 2016). The relatively higher composition of hydrophilic BAs in rodents (muricholic BAs and their taurine

conjugates) induces less hepatocyte toxicity than human BAs (Oizumi et al. 2017).

Due to the biological and clinical significance of BA, several bioanalytical methods were established, particularly during the last two decades. These analytical platforms are based on the separation of BA, their identification, and quantification from different biological samples. The most popular techniques used for BA quantitation in clinical laboratories are enzymatic assays, liquid-/gas-chromatography (GC), and mass spectrometry (MS). The application of enzymatic assays offers the possibility for measuring the amount of total bile acids (TBA) in plasma and serum. These assays are simple in use, rapid, suitable for automation, and inexpensive. The enzyme 3- α -hydroxysteroid dehydrogenase (3- α -HSD) converts bile acids to 3-keto steroids and Thio-NADH in the presence of Thio-NAD. The formation of Thio-NADH can be determined spectrophotometrically at 405 nm (Zhang et al. 2005). Another assay is based on the formation of formazan dye and can be performed at 540 nm. Here, the enzyme 3- α -HSD converts bile acids to 3-ketosteroids and NADH in the presence of NAD. The resulting NADH reacts with nitro-tetrazolium blue (NBT) to form a formazan dye in the presence of diaphorase enzyme (Mashige et al. 1981). However, measurements of individual BA with these assays, as, e.g., primary and secondary BA or taurine and glycine groups, are not possible. The use of HPLC (high-performance liquid chromatography) for separation of BA compounds in combination with UV-detection can be performed after pre-column derivatization with, e.g., nitrophenacylbromide and detection at a wavelength of 263 nm (Shi et al. 2015). This method is more suitable for very high concentrations of BA in biological samples. GC-MS separation and quantitation of BA is quite sensitive but needs sample derivatization steps and deconjugation reactions due to the BAs' thermal instability and low volatility (Griffiths and Sjovald 2010). In the field of BA profiling in biological matrices, UPLC-MS/MS (ultra-performance liquid chromatography coupled to tandem mass spectrometry) is considered the analytical technique of choice nowadays (Garcia-Canaveras et al. 2014). UPLC has a much higher separation efficiency than HPLC due to < 2 μ m particle size technology of the stationary phases and faster elution of the analytes. Identification and quantification of BA, including their derivatives, are conducted with electrospray ionization source in negative ion mode and can be performed in single-ion monitoring (SIM), single-reaction monitoring (SRM), and multiple-reaction monitoring (MRM) modes to increase the selectivity and sensitivity (Yuan et al. 2012). The isobaric masses (same molecular ion/fragment ion) such as TDCA, TCDCA, THDCA, and

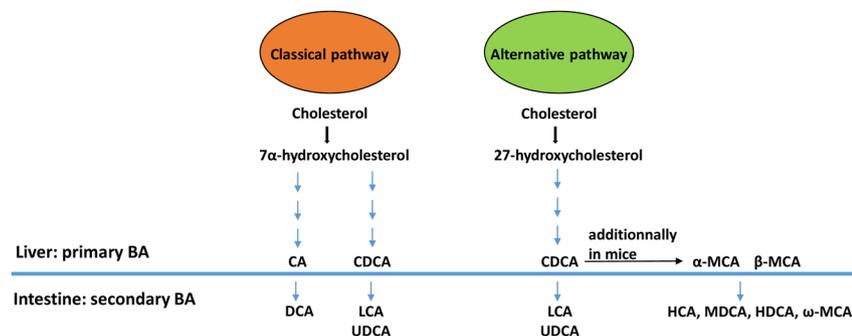


Figure 1: Synthesis of primary and secondary bile acids in the liver and intestine. BA (bile acids), CA (cholic acid), CDCA (chenodeoxycholic acid), DCA (deoxycholic acid), LCA (lithocholic acid), UDCA (ursodeoxycholic acid), MCA (muricholic acid), HCA (hyocholic acid), HDCA (hyodeoxycholic acid), and MDCA (murideoxycholic acid).

TUDCA (m/z 498/80) are not distinguishable with tandem MS. Hence, there exists a need for an excellent chromatographic separation with UPLC to assign the proper retention times of each compound. In this way, the combination of UPLC and MS/MS data offers an accurate quantification and confirmation assay. Bile acid profiles can serve as diagnostic markers, e.g., for inborn errors of bile acid metabolism (Herebian and Mayatepek 2011) and different liver or gastrointestinal diseases (Sarafian et al. 2015; Sugita et al. 2015). Therapeutic drug monitoring of UDCA and its derivatives GUDCA/TUDCA in patients with cholestatic diseases is also of clinical significance (Guarino et al. 2013; Lazaridis et al. 2001).

Bile salt transport in the human liver

BA are synthesized *de novo* from cholesterol (Figure 2) in the cytosol of hepatocytes. However, they perform their function to solubilize hydrophobic compounds such as vitamins in the intestine. Interestingly, approximately 95% of the BA undergo enterohepatic circulation (Hofmann 1984; Keitel et al. 2019a; Ridlon et al. 2006) meaning they are transported back to the liver and re-absorbed by hepatocytes. The main player for the re-uptake of BA across the basolateral membrane of hepatocyte is the sodium taurocholate co-transporting polypeptide (NTCP or SLC10A1), which is a member of the solute carrier family (SLC) and was first cloned and characterized in 1991 (Hagenbuch et al. 1991). Taurocholate (TC)/sodium is transported in a symport fashion and at a stoichiometry of 1:2. However, NCTP not only imports TC, but all BA as well as steroids, thyroid hormones, and certain xenobiotics (Jung et al. 2004). Importantly, NTCP acts as a receptor for the hepatitis B and D viruses and has turned into an important target for drug development (Bogomolov et al. 2016; Ni et al. 2014). Unfortunately, no structural information for NTCP exists, and the bacterial paralogue from

N. meningitidis, ASBT_{NM}, for which there is a crystal structure (Hu et al. 2011), cannot directly be used as a template for structural modeling since the two interaction sites of NTCP (amino acids 84–87 and 157–165) of the hepatitis B virus are very divergent in sequence in different species (Yan et al. 2013). Nevertheless, the bile salt and sodium ion binding sites of NTCP and ASBT_{NM} are highly conserved (Hu et al. 2011). NTCP is capable of importing all BA. However, an alternative import route is provided by the three OATPs (organic anion transport proteins, OATP1B1, OATP1B3, and OATP2B1) (Konig et al. 2000). Their substrate spectrum is much larger than BA only, and the transport occurs in a Na⁺-independent manner (Hagenbuch and Meier 2004), but together with bicarbonate (Leuthold et al. 2009).

In the canalicular membrane, BA import is not occurring, rather export into the bile duct takes places and is mediated by ABCB11 (BSEP (bile salt export pump)) or s-Pgp (sister of P-gp (P-glycoprotein, MDR1, ABCB1)), which constitutes the major bile salt export pathway. ABCB11 was first described in 1995 and initially termed sister of P-gp due to the high sequence identity of ABCB1, a classic drug export pump, and ABCB11 (Childs et al. 1995). Only in 1998, its natural function as a bile salt export pump especially for glycine and taurine conjugated BA, although with a preference for taurine-conjugated BA, was demonstrated for rat ABCB11 (Gerloff et al. 1998). A similar preference was observed for mouse and human ABCB11 (Byrne et al. 2002; Hayashi et al. 2005; Noe et al. 2001; Noe et al. 2002). Equally important, mutations of ABCB11 were identified as the molecular reason for progressive familial intrahepatic cholestasis type 2 (PFIC2) (Strautnieks et al. 1997).

Human ABCB11 represents the rate-limiting step of BA secretion in hepatocytes (Stieger 2009). This makes it an interesting target of functional and structural studies. However, cloning of the ABCB11 gene has been extremely challenging and time-consuming (Byrne et al. 2002; Noe et al. 2002). One of the many problems encountered is the instability and integrity of the gene in *E. coli*. Therefore,

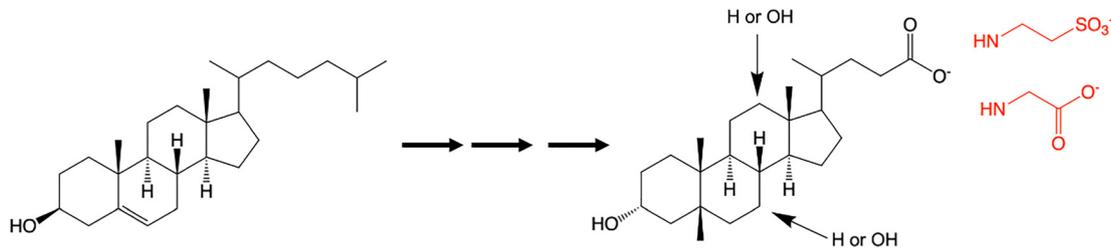


Figure 2: Chemical structure of cholesterol (left) and bile salts (right). The modification present in different bile salts are indicated (position 3 (CH_3^-), 7 (H or OH), and 12 (H or OH)), while the two forms of conjugation (taurine or glycine), which increase solubility, are shown in red.

Stindt et al. (Stindt et al. 2011) developed a yeast-based cloning procedure for ABCB11 that completely bypassed *E. coli* and allowed the subsequent overexpression and purification of ABCB11 in the yeast *P. pastoris* (Ellinger et al. 2013). *P. pastoris* had been already used to produce other human ABC transporters (Chloupkova et al. 2007) and was also successfully used for ABCB11 (Ellinger et al. 2013). In contrast to (over)expression in insect cells (Byrne et al. 2002; Noe et al. 2002), this system allowed to purify ABCB11 based on the speed of cloning and ease of handling and study clinically relevant mutants of ABCB11 *in vitro* under defined conditions (Ellinger et al. 2017; Stindt et al. 2013). These studies even allowed determination of the minimal number of BA transported by ABCB11, which were eight BA per second and transporter (Ellinger et al. 2017). With a yeast-based expression system at hand, the studies were moved one step further, and the interactome of ABCB11 was determined employing the membrane yeast two-hybrid (MYTH) approach (Stagljar et al. 1998) using a cDNA library of human liver membrane proteins as well as soluble proteins. Positive interaction partners of ABCB11 determined by MYTH were subsequently verified by *in vitro* interaction studies, which resulted in the identification of 10 interaction partners. This included, e.g., radixin, a protein that cross-links the membrane and the cytoskeleton (Clucas and Valderrama 2014) or the bile acyl-CoA synthetase (BACS or SLC27A5) (Mihalik et al. 2002). Surprisingly, the scaffolding protein NHERF1, a known interaction partner of CFTR/ABCC7, MRP2 (ABCC2), and MRP4 (ABCC4) (Hoque et al. 2009; Li et al. 2010; Short et al. 1998) was not identified in this screen. In summary, the yeast-based overexpression proved to be a valuable tool to rapidly screen clinically relevant mutations, not only of ABCB11, but also of ABCB4 (Dzagania et al. 2012; Kluth et al. 2015). ABCB4 is a lipid-, more precisely, a phosphatidylcholine-specific floppase and will be discussed further at the end of this section. PC next to BA and cholesterol are the major components of bile. Thus, as one has to expect that BA transport by ABCB11 and PC flopping by ABCB4 are coupled,

incubation of rat liver vesicles with TC increased PC flopping mediated by ABCB4 (Gerloff et al. 1998).

The single-particle cryo-electron microscopy (EM) structure of ABCB11 (Wang et al. 2020) in the so-called inward-facing conformation, i.e., where the binding site is accessible from the cytosol, was determined in the absence of substrate and nucleotide, respectively (Figure 3). The overall architecture is, as expected, identical to other known structures of the ABCB subfamily (for a recent review see (Locher 2016)). Thus, the transmembrane domains (TMDs) are split into two parts, a domain swapping, which was first observed in the crystal structure of Sav1866 (Dawson and Locher 2006) and connects both nucleotide-binding domains (NBDs) through the so-called coupling helices (CHs; blue and green in Figure 3). In contrast to human ABCB1 (Alam et al. 2019), the N-terminus of ABCB11 is extended. In the structure, this extension adopts a helical

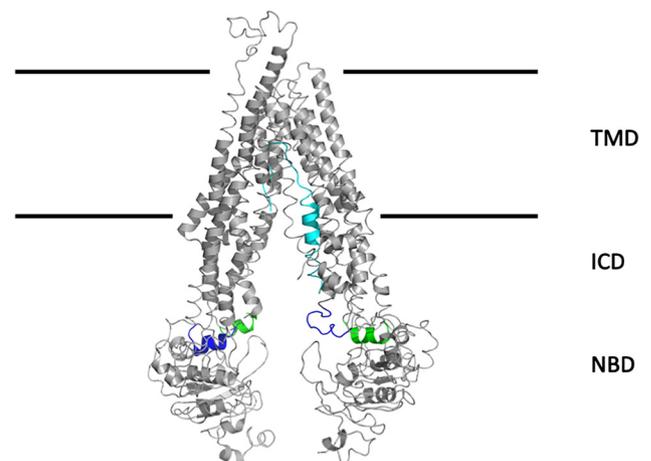


Figure 3: Structure of ABCB11 in cartoon representation. The putative position of the membrane is indicated by the two horizontal lines. The individual domains are labeled (ICD: intracellular domain, TMD: transmembrane domain, NBD: nucleotide-binding domain). The N-terminal extension of ABCB11 is highlighted in cyan and the coupling helices of TMD1 in green and TMD2 in blue.

fold that protrudes into a cavity that is accessible from the cytosol and harbors the putative ligand binding site (cyan in Figure 3). Such an N-terminal extension was also observed in the ABCB1 orthologue from *C. elegans* (Jin et al. 2012). However, its functional role remained unclear. Deletion of the N-terminal extension in ABCB1 did not influence function; rather, the expression level was two-fold reduced, pointing to a role in trafficking and/membrane insertion. The structure of ABCB1 (Wang et al. 2020) represents an important achievement. However, additional structures, especially in the presence of BA, are required to understand the transport mechanism of ABCB1.

The expression of ABCB1 is regulated in many ways. The most important regulator is the farnesoid X receptor (FXR, NR1H4) (Lefebvre et al. 2009), which regulates not only the expression of ABCB1, but also NTCP and the enzymes of the bile salt synthesis pathway (Eloranta and Kullak-Ublick 2005). Importantly, the regulation of ABCB1 occurs not only on the level of expression. In parallel, a short-term regulation of hepatobiliary transport involves rapid insertion and retrieval of transporters, such as Bsep, Mrp2, and Ntcp into or from the canalicular or sinusoidal membrane, respectively (Anwer et al. 2005; Beuers et al. 2001; Cantore et al. 2011; Kubitz et al. 1997; Kurz et al. 2001; Mayer et al. 2019; Mukhopadhyay et al. 1997; Schmitt et al. 2001; Sommerfeld et al. 2015; Webster et al. 2000). This was extensively studied in isolated perfused rat liver employing hypo- and hyperosmotic perfusion conditions. Shifting the ambient osmolarity from 305 to 225 mosmol/L doubled within minutes the taurocholate excretion capacity into bile, whereas hyperosmolarity (385 mosmol/L) was cholestatic (Häussinger et al. 1992). As shown by immunohistochemistry, these osmolarity shifts were accompanied a rapid and reversible insertion of Abcb11 and Abcc2 (Mrp2) into the canalicular membrane in response to hypoosmotic cell swelling, whereas hyperosmotic hepatocyte shrinkage triggered the endocytosis of these transporters into a subcanalicular vesicular compartment (Cantore et al. 2011; Kubitz et al. 1997; Schmitt et al. 2001). Hyperosmotic hepatocyte shrinkage also induced retrieval of Ntcp from the sinusoidal membrane (Sommerfeld et al. 2015), whereas hypoosmotic hepatocyte swelling stimulated the insertion of Ntcp into the sinusoidal plasma membrane (Webster et al. 2000). Thus, sinusoidal uptake and canalicular excretion of bile acids are coordinately regulated by ambient osmolarity, most likely representing a mechanism to avoid proapoptotic bile acid overload of the hepatocyte. The signal transduction pathways leading to hyperosmotic Ntcp and Abcb11 and Abcc2 retrieval from the sinusoidal and canalicular membrane, respectively, have been identified. Here, activation of the Src family kinase Fyn by

NADPH oxidase-derived reactive oxygen species (ROS) is a crucial event, whereas the parallel activation of the Src family kinase member Yes triggers a proapoptotic state in response to hepatocyte shrinkage (for review see Reinhr et al. 2013). Also, oxidative stress exerted by glycochenodeoxycholate (Mayer et al. 2019) or t-butyl hydroperoxide (Schmitt et al. 2000) triggers retrieval of Abcb11 and Abcc2 from the canalicular membrane. On the other hand, hypoosmotic hepatocyte swelling leads to an integrin-dependent c-Src activation, which triggers activation of Erks and p38^{MAPK} (Häussinger et al. 2003) and probably involves the p38 α isoform (Schonhoff et al. 2016). Dual activation of these MAP-kinases is required for insertion of Abcb11 and Abcc2 into the canalicular membrane. Interestingly, the hypoosmotic signaling pathway towards choleresis is also activated by tauroursodeoxycholate, which can non-osmotically activate $\alpha_5\beta_1$ integrins (Gohlke et al. 2013). Hypoosmotic insertion of Ntcp into the sinusoidal membrane involves activation of PI3 Kinase and protein kinase B (Webster et al. 2000; Webster et al. 2002).

Thus, the vesicular pool of ABCB11, similar to other hepatobiliary transporters, can react fast to external and internal stimuli that require an increase or decrease of the BA levels in the bile duct.

However, ABCB11 is not an isolated entity. Rather, BA secretion by ABCB11 is coupled to cholesterol transport by ABCG5/G8 (Berge et al. 2000) and phosphatidyl (PC) lipid flop from the inner to the outer leaflet of the canalicular membrane (van Helvoort et al. 1996). The latter process is catalyzed by ABCB4 (MDR3) (Prescher et al. 2019). The molecular reason for such a 'bile triumvirate' (Kroll et al. 2021) is the harsh detergent action of BA. Thus, the formation of mixed micelles composed mainly of BA, PC lipids, and cholesterol, i.e., bile, diminishes the detergent action of BA and prevents the solubilization of the canalicular membrane and the formation of gallstones (Small 2003). Another factor contributing to the resistance of the canalicular membrane against the detergent action of BA, is the asymmetric distribution of lipids between the two leaflets of the lipid bilayer. ATP8B1 flips phosphatidylserine from the outer into the inner leaflet of the membrane, counteracting the action of ABCB4 (Groen et al. 2011). Absence of ATP8B1 reduces the resistance of the canalicular membrane towards BA and impairs BA transport (Paulusma et al. 2006). In humans, mutations in the ATP8B1 gene are associated with hereditary intrahepatic cholestasis ranging from intrahepatic cholestasis of pregnancy (ICP) to progressive familial intrahepatic cholestasis (PFIC) (see below) (Bull et al. 1998; Van Mil et al. 2007; van Mil et al. 2004).

Bile is composed of approximately 70% bile salts, 20% PC lipids, and 8–9% cholesterol in healthy adults (Fisher

and Yousef 1973). This cross-communication is also reflected by the fact that bile salts increase the secretion of PC lipids by ABCB4, which requires not only the choline headgroup, but also two acyl chains for proper recognition of the PC lipid (Prescher et al. 2020). A similar observation was also made for ABCG5/G8, which completely lacks ATPase activity in the absence of BA (Johnson et al. 2010). On the other hand, the concentration of cholesterol in the membrane modulates the activity of ABCB11 (Guyot et al. 2014; Kis et al. 2009).

In summary, BA transport is not a simple import by Ntcp, diffusion through the cytosol or intracellular lipid membranes and followed by a simple export by ABCB11. Instead, a complex network of interactions and crosstalk between the different transporters is apparently present, which regulates and modulates the amount of bile salts in the hepatocyte and the bile duct. So far, we have only seen the tip of the iceberg. More studies are required to unravel the molecular signals and mechanisms that allow fine-tuned bile salt transport.

Hereditary disorders of bile salt transport

At the canalicular membrane, ABCB11, ABCB4, and the heterodimeric cholesterol transporter ABCG5/8 ensure the coordinated excretion of BA, phospholipids, and cholesterol, respectively, into bile. Within bile, these molecules assemble to form mixed micelles (Admirand and Small 1968), enabling the elimination of cholesterol. Approx. 12–20 g of BA have to be excreted per day into bile to eliminate 500 mg of cholesterol.

Canalicular secretion is regarded as the rate-limiting step of the vectorial transport of bile acids across hepatocytes (Häussinger et al. 2000; Trauner and Boyer 2003). Disturbance of bile salt transport results in elevated BA concentrations in different compartments and may exert toxic effects due to their detergent and proapoptotic properties (Reinehr et al. 2003). Bile salt retention within hepatocytes is directly or indirectly involved in drug-induced liver injury (DILI) (Morgan et al. 2010; Ogimura et al. 2011). These properties explain why ABCB11 and cooperating canalicular transporter proteins are associated with hereditary liver diseases. These diseases comprise progressive familial intrahepatic cholestasis (PFIC) type 1 to 3, benign recurrent intrahepatic cholestasis (BRIC) type 1 and 2, low phospholipid associated cholelithiasis (LPAC), and intrahepatic cholestasis of pregnancy (ICP) (Figure 4). Here we will focus on ABCB11-associated diseases.

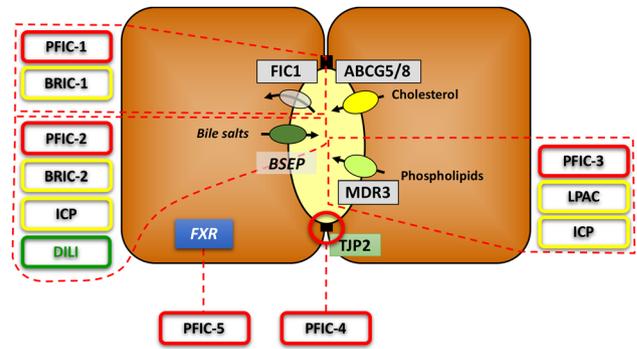


Figure 4: Hereditary cholestatic liver diseases. Within this model of a hepatocyte couplet several proteins are shown, which are associated with distinct cholestatic liver diseases. Severe diseases, called progressive familial intrahepatic cholestasis (PFIC) type 1 to 5, have been identified. PFIC-1 as well as benign recurrent intrahepatic cholestasis (BRIC-1) are linked to the flippase ATP8B1 (FIC1), which translocates phosphatidylserine and phosphatidylethanolamine from the outside to the inner leaflet of the membrane bilayer. The ABC transporter ABCB11 (BSEP) and ABCB4 (MDR3) are also related to diseases of varying severity and symptoms such as intrahepatic cholestasis of pregnancy (ICP) or low phospholipid associated cholelithiasis (LPAC). Certain polymorphisms of ABCB11 (BSEP) are not harmful per se but the liver may be more susceptible to injury due to certain drugs. More recently, the tight junction protein 2 (TJP2) and the nuclear BA receptor FXR (NR1H4) were identified as disease-relevant.

FIC1, familial intrahepatic cholestasis protein 1; BSEP, bile salt export pump; MDR3, multidrug resistance 3; ABCG5/8, ATP-binding cassette transporter G5/G8; TJ2, tight junction protein 2; FXR, Farnesoid X receptor; PFIC, Progressive familial intrahepatic cholestasis; BRIC, Benign recurrent intrahepatic cholestasis; ICP, Intrahepatic cholestasis of pregnancy; LPAC, phospholipid associated cholelithiasis; DILI, drug induced liver injury.

Progressive familial intrahepatic cholestasis type 2 (PFIC-2)

Progressive familial intrahepatic cholestasis type 2 (PFIC) describes a cholestasis-induced end-stage liver disease in childhood, which is distinct from other neonatal or pediatric cholestatic diseases such as biliary atresia or Alagille syndrome (Ballou et al. 1973). Biochemically, PFIC-2 is characterized by low gamma-glutamyltranspeptidase (gGT) levels and is linked to mutations of ABCB11/BSEP (Bull et al. 1997). In contrast to PFIC-2 (and PFIC-1 due to mutations of FIC1), PFIC-3 is characterized by increased gGT levels and is due to mutations of ABCB4/MDR3. In the case of PFIC-2, normal gGT levels are attributed to the low concentrations of BA in bile and, consequently, reduced damage to cholangiocytes (Jacquemin 2001). PFIC-2 patients present within the first year of life jaundice, itching, growth failure, and occasionally severe hemorrhage due to

vitamin K deficiency. Importantly, PFIC-2 patients have a high risk to develop hepatocellular (HCC) (Knisely et al. 2006) or cholangiocellular carcinoma (CCA) (Strautnieks et al. 2008), and an association of the common BSEP polymorphism p.V444A has been described in a study of 172 CCA-patients (Wadsworth et al. 2011).

Reduced or even absent expression of canalicular BSEP depends on the type of genetic variant (Kubitz et al. 2014) and may be utilized for diagnostic purposes (Evason et al. 2011; Keitel et al. 2005; Strautnieks et al. 2008). Nowadays, more than 150 different variants have been associated with a PFIC-2 phenotype (<http://www.hgmd.cf.ac.uk>) (Droge et al. 2017; Kroll et al. 2021).

The reason for the high rate of absent BSEP in immunohistochemical studies is discussed elsewhere (Kubitz et al. 2014; Strautnieks et al. 2008). Interestingly, BSEP seems to be prone for degradation within the ER-associated degradation (ERAD) pathway as shown in expression studies of cloned BSEP containing the very common polymorphism p.V444A and the common PFIC-2-related variants p.E297G and p.D482G as compared to wildtype BSEP (Hayashi and Sugiyama 2009; Keitel et al. 2009).

Benign recurrent intrahepatic cholestasis type 2 (BRIC-2)

Some ABCB11-mutations are linked to milder forms of cholestasis (Kubitz et al. 2006), which typically resolve spontaneously. It is termed ‘benign recurrent intrahepatic cholestasis type 2’ (BRIC-2) (van Mil et al. 2004) and clinically presents similar to BRIC-1 (Brenard et al. 1989), which is caused by ‘mild’ variants of ATP8B1 (FIC1) (Bull et al. 1998). FIC1 or familial intrahepatic cholestasis 1 protein is encoded by the ATP8B1 gene (Bull et al. 1998) and serves as a flippase, which transports phosphatidylserine or phosphatidylcholine (Groen et al. 2011; Shin and Takatsu 2019).

BRIC-2 patients have a higher incidence of gallstones than BRIC-1 patients (van Mil et al. 2004). The penetrance of single ABCB11 variants in BRIC-2 is variable as shown in siblings with the ABCB11-mutation p.G374S: while the brother developed liver cirrhosis, the sister experienced self-limiting jaundice with pruritus (Stindt et al. 2013).

Intrahepatic cholestasis of pregnancy and bile salt export pump (BSEP)

Intrahepatic cholestasis of pregnancy (ICP) is diagnosed in the absence of any other liver disease and presence of pruritus and elevated serum BA levels (European Association for

the Study of the 2009). Typically, ICP occurs during the third trimester, and liver functions tests (AST/GOT and ALT/GPT) are slightly elevated in most cases but may rise up to 50-fold over the upper limit of normal, thereby resembling liver failure (Keitel et al. 2006; Keitel et al. 2016; Lammert et al. 2003; Rioseco et al. 1994; Williamson and Geenes 2014). Jaundice is observed in up to 10% of ICP cases. Increased serum BA concentrations are almost diagnostic for ICP. BA levels above 40 $\mu\text{mol/l}$ are predictive for ICP-related complications, such as preterm birth or asphyxia, while stillbirth risk is increased in women with BA levels above 100 $\mu\text{mol/l}$, especially after the 35th week of gestation (Glantz et al. 2004; Ovadia et al. 2019). ICP almost invariably resolves spontaneously within 2–4 weeks after delivery; otherwise differential diagnoses have to be taken into account.

Anti-bile salt export pump (BSEP) antibodies and cholestasis

Some PFIC-2 patients may not be sufficiently treated by medical (e.g., UDCA) or surgical (e.g., partial biliary external diversion (PEBD) interventions and require liver transplantation (van Wessel et al. 2020). It has been recognized that 8–33% of PFIC-2 patients develop recurrent cholestasis similar to preceding PFIC-2 following liver transplantation (Droge et al. 2017; Jara et al. 2009; Keitel et al. 2009; Siebold et al. 2010; Stindt et al. 2016). *De novo* formation of anti-BSEP antibodies, which bind to an extracellular loop of the N-terminal half of BSEP and inhibit BSEP transport activity, have been identified as the underlying cause of antibody-induced BSEP deficiency (AIBD) (Keitel et al. 2009; Stindt et al. 2016). It is likely that the complete absence of ABCB11 expression (e.g., due to Stop-mutation) in the original liver prevents auto-tolerance towards ABCB11 and therefore facilitates antibody-formation after transplantation (Kubitz et al. 2015). Treatment of these children includes changes of the regimen of immunosuppressant, plasmapheresis, immune-adsorption, B-cell depletion by rituximab, or even allogeneic haematopoietic stem cell transplantation in order to remove ABCB11-reactive plasma cells (Brinkert et al. 2018; Keitel et al. 2009; Kubitz et al. 2015).

Bile salt signaling in the human liver

While some of the functions of the nuclear BA receptor FXR (NR1H4) for hepatic BA transport were discussed above, we will focus in the following paragraphs on the G-protein coupled BA receptor TGR5.

TGR5 – a bile acid receptor

TGR5 (Kawamata et al. 2003; Keitel and Häussinger 2018; Maruyama et al. 2002), also known as G protein-coupled bile acid receptor 1 (GPBAR1) or membrane-type bile acid receptor (M-BAR), was the first GPCR identified responsive to BA in 2002/2003 (Kawamata et al. 2003; Maruyama et al. 2002). TGR5 mRNA is almost ubiquitously expressed in rodent and human tissues (Keitel and Häussinger 2012, 2018; Maruyama et al. 2002; Maruyama et al. 2006; Reich et al. 2017), with high expression found in organs involved in the enterohepatic circulation and excretion of BAs such as liver, gallbladder, and small and large intestine (Kawamata et al. 2003; Keitel et al. 2010; Keitel et al. 2013; Maruyama et al. 2002; Maruyama et al. 2006; Vassileva et al. 2006). Moreover, TGR5 mRNA was also present in heart, lung, spleen, CD14-positive monocytes, placenta, female and male reproductive organs, adrenal glands, brain, and adipose tissue (Kawamata et al. 2003; Keitel et al. 2010; Keitel et al. 2013; Maruyama et al. 2002; Maruyama et al. 2006; Vassileva et al. 2006). Among other physiological functions, TGR5 has been shown to influence glucose tolerance via GLP-1 secretion and protect bile duct epithelial cells from BA-induced toxicity (Merlen et al. 2020; Reich et al. 2017; Thomas et al. 2009; Velazquez-Villegas et al. 2018).

TGR5 recognizes a broad spectrum of hydrophobic endogenous ligands (Sato et al. 2008), comprising all known BAs and many hydrophobic neurosteroids, such as estradiol, pregnanediol, and allopregnanolone (Keitel et al. 2010; Martin et al. 2013; Sato et al. 2008) (Figure 5). In contrast to other BA-sensing receptors, TGR5 is rather promiscuously sensing BA regardless of their substitution pattern and conjugation state (Sato et al. 2008). This underlines the role of TGR5 as a protective mechanism against the toxicity of BA for gallbladder epithelial cells and cholangiocytes (Beuers et al. 2010; Deutschmann et al. 2018; Reich et al. 2016, 2017). Thus, Tgr5-deficient animals are more susceptible to BA-induced liver damages (e.g., BA feeding, common bile duct ligations (Klindt et al. 2019; Reich et al. 2016)). Conversely, overexpression of Tgr5 ameliorates the activated biliary phenotype characteristic of sclerosing cholangitis induced by Abcb4-deficiency (Reich et al. 2021). Tgr5 expression levels are reduced in cholangiocytes of Abcb4-deficient mice and can be increased/restored by UDCA/nor-UDCA feeding, respectively (Reich et al. 2021). However, overexpression of TGR5 has been observed in human cholangiocarcinoma (Deutschmann et al. 2018; Erice et al. 2018; Reich et al. 2016).

TGR5 binding mode model

BA activate TGR5 via the orthosteric ligand binding site (Gertzen et al. 2015). There, BA bridge the transmembrane helices (TM) three and six to activate the receptor, as is common for GPCR agonists (Katritch and Abagyan 2011). Further TMs, particularly TM5, are addressed to increase the efficacy (Gertzen et al. 2015) (Figure 6(A) and (B)). Taurine conjugation increases the size of a BA, which allows forming a likely affinity-increasing salt-bridge interaction to R79 (EL1), while a hydrogen bond to the vital Y240 (TM6) in TGR5 is still possible (Gertzen et al. 2015). All BA employ the 3-hydroxy groups of their cholane scaffold to form a hydrogen bond to Y240, and this interaction is further stabilized by a hydrogen bond to E169 (TM5, Figure 6(A) and (B)) (Gertzen et al. 2015). Agonistic neurosteroids such as pregnanediol also utilize their hydroxy or carbonyl groups to interact with Y240 in TGR5 (Gertzen et al. 2015). Lacking acidic groups, they mainly form additional hydrophobic interactions with Y89 in TM3 to bind to and activate TGR5 at a reasonable EC_{50} (e.g., pregnanediol $EC_{50} = 0.58 \mu\text{M}$ (Sato et al. 2008) (Figure 5), allowing them to activate TGR5 in the brain. The epimeric selectivity has been explained by a hydrogen bond formation of CDCA's 7 α -hydroxy group to Y89 in TM3 of TGR5, which is not possible in the β -configured UDCA. A recently discovered cholic acid 7-sulfate (CA7S, Figure 5) (Chaudhari et al. 2021), in which the 7-hydroxy group is esterified with sulfuric acid, could also form a hydrogen bond.

Synthetic TGR5 agonists and antagonists

Currently, various TGR5 agonists with a non-steroidal core are available (Cao et al. 2016; Duan et al. 2015) (Figure 5). Here, an acidic or amide moiety connects with three to four variably interconnected aromatic and aliphatic rings. The ring furthest from the acid or amide moiety is heteroaromatic or -aliphatic. Although the binding mode of non-steroidal TGR5 agonists has not been investigated to date, it is possible that the heteroatom mimics the 3-hydroxy group of bile acids and, thus, forms the crucial hydrogen bond to Y240 (TM6) (Spomer et al. 2014). Intestinal specificity can be achieved by either introducing a permanent positive charge via a quaternary ammonia moiety to the synthetic TGR5 agonists (**26a**, Figure 5) or creating a higher-molar mass agonist via linking two agonists through polyethylene glycol (**15c**, Figure 5).

As a wide range of hydrophobic substances activates TGR5, it is not trivial to develop a ligand that binds to TGR5

| Bile acid | R ₁ | R ₂ | R ₃ | EC ₅₀ [μM] |
|-----------|----------------|----------------|----------------|-----------------------|
| TLCA | H | H | Taurine | 0.29 |
| DCA | H | α-OH | OH | 1.25 |
| CDCA | α-OH | H | OH | 6.71 |
| UCDA | β-OH | H | OH | 36.40 |
| TCA | α-OH | α-OH | Taurine | 4.95 |
| LCA | H | H | OH | 0.58 |
| CA7S | α-Sulfate | α-OH | OH | 0.17 |

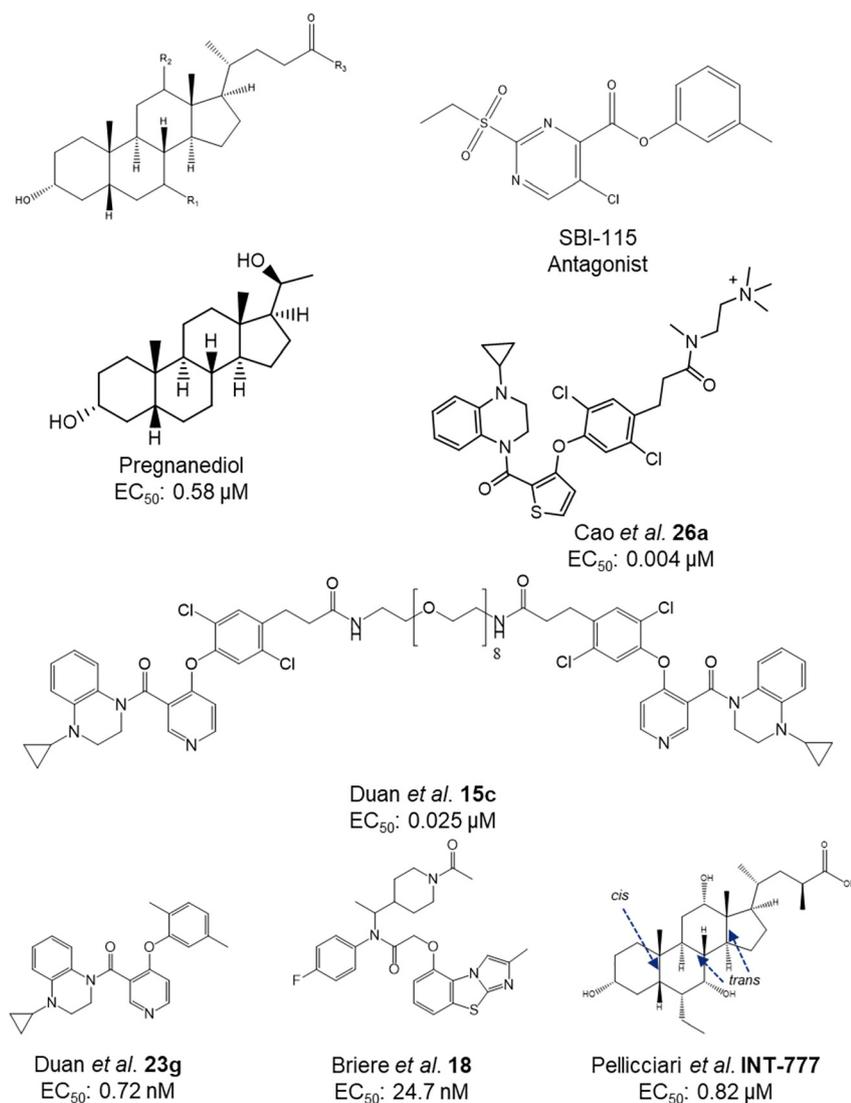


Figure 5: Important TGR5 ligands. The table shows the substitution pattern for selected natural bile acid agonists with their EC₅₀ values (Sato et al. 2008).

In addition, a neurosteroid agonist, pregnanediol, two intestinal agonists, 26a and 15c, three synthetic agonists, 23g, 18, and INT-777, and an antagonist, SBI-115, are shown. Additionally, the *cis* and *trans* configurations between the rings of the cholane scaffold of INT-777 are indicated by blue dashed arrows.

but does not activate it. Hence, only one antagonist has been discovered (SBI-115, Figure 5) (Masyuk et al. 2017) so far. It is composed of two interconnected aromatic rings and an ethyl sulfo substituent and is slightly smaller than the known agonists. Due to the lack of an acid moiety and a steroidal core, the binding mode of this antagonist cannot easily be inferred from the agonist binding modes.

Recently, a cryo-EM structure of TGR5 in complex with INT-777 (6α-ethyl-23(S)-methylcholic acid (S-EMCA), Figure 5), a potent and selective TGR5 agonist with *in vivo*

activity (Pellicciari et al. 2009), has been resolved (PDB-ID: 7CFN) (Yang et al. 2020). In contrast to all other crystallized GPCRs, the conserved tryptophane residue in TM6 (W237), which likely functions as a ligand-dependent trigger for GPCR activation (Katritch et al. 2013), is located one helix turn further down to the cytoplasm, suggesting that the ligand-dependent activation of TGR5 is carried out differently. Unexpectedly, all rings of the cholane scaffold of INT-777 modeled in the complex structure are connected in a *trans* configuration, although the configuration of the

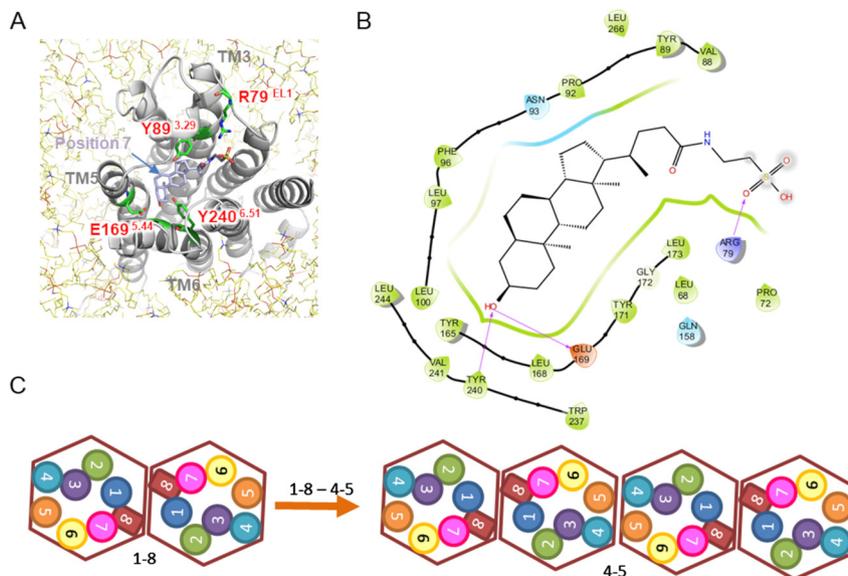


Figure 6: Interactions of TGR5 with TLC and TGR5 oligomer formation.

(A) Binding mode model of TLC (light blue sticks) in TGR5 (grey, cartoon), embedded in a lipid bilayer (yellow, lines) (Gertzen et al. 2015). Important interacting residues are shown as green sticks, and position 7 of the cholane scaffold is indicated by an arrow. TLC bridges TM3 and TM6 by interacting with Y89 and Y240, which are important for TGR5 activation. The interaction with E169 is suggested to contribute to the efficacy of TLC. (B) 2D-diagram showing interactions of TLC within TGR5. Hydrogen bonding and salt-bridge interactions are shown as arrows, hydrophobic interactions are shown in green. (C) Schematic of TGR5 oligomerization (Greife et al. 2016; Wäschenbach et al. 2020). TGR5 forms dimers involving TM1 and helix 8 (1–8). Then, oligomerization can occur from dimers via the TM4–TM5 interface (4–5).

5 β -cholane scaffold of **INT-77** is *cis-trans-trans* (Figure 5) (Pellicciari et al. 2009). As it is not clear to what extent the shown binding mode of **INT-77** within TGR5 is impacted by this change, it cannot be used to interpret the epimeric selectivity of TGR5 agonists, particularly between CDCA and UDCA (Sato et al. 2008), and mutational data (Gertzen et al. 2015). Furthermore, note that in Figure 4E of Ref. (Yang et al. 2020), the configurations of chiral centers of the chain and the cholane scaffold are inverted with the exception of C17, and a methyl group has been added to C5. Here, in contrast to the modeled **INT-77**, the connectivity of the rings is *cis-trans-trans*.

Regulation of TGR5 through di- and oligomerisation

GPCR oligomerization has been shown to influence various biological functions (Hiller et al. 2013). For TGR5, a combined strategy using cell biology, multiparameter image fluorescence spectroscopy (MFIS) for quantitative FRET analysis, and integrative modeling revealed the oligomerization properties of TGR5, fused to fluorescent proteins, in live cells (Greife et al. 2016). The correlation of FRET parameters with increasing acceptor dye-to-donor dye ratios showed that TGR5 wildtype forms higher-order oligomers. A TGR5 Y111A variant inhibits oligomerization but still allows TGR5 dimerization. From the ratio dependence, it was concluded that higher-order oligomers of TGR5 wildtype are formed from dimers interacting with other dimers.

Subsequent integrative modeling considering the energetic preference of dye positions showed that higher-order oligomers are linear arrangements of dimers interacting via TM1 and helix 8, with the oligomerization site involving TM4 and TM5 (Figure 6(C)). Extensive energetic evaluation of GPCR dimerization on the atomistic level showed that the formation of both the 1–8 and 4–5 interface is energetically favorable for TGR5 (Wäschenbach et al. 2020). The formation of the 1–8 interface is favored over that of the 4–5 interface, and differences in lipid-protein interactions contribute to the observed differences in free energies of association. A membrane-proximal, C-terminal helix is structurally required for plasma membrane localization and function of TGR5 (Spomer et al. 2014). Together with the finding that helix 8 is important for TGR5 dimerization, this may imply that dimerization of TGR5 is necessary for its membrane trafficking. Finally, time series MFIS-FRET analysis before, at the time point of, and after stimulation of TGR5 with taurocholate (TCA, Figure 6), a bile acid less cytotoxic than TLCA in live cells, indicated that TCA, and subsequent G protein coupling, does not influence the oligomerization state of TGR5 wildtype and Y111A (Greife et al. 2016), an observation supported by the literature (Patowary et al. 2013).

In summary, TGR5, a GPCR involved in bile duct epithelium protection, among other physiological functions, but which may also contribute to tumor formation, is activated by a wide variety of hydrophobic ligands. By contrast, only one antagonist is known to date. TGR5's function and membrane localization can be regulated

through di- and oligomerization. TM1 and helix 8 as well as TM4 and TM5 constitute the respective interfaces. While recent clinical trials could not establish TGR5 agonists as drugs for diabetes treatment, potent antagonists could be beneficial in treating cholangiocarcinomas.

Summary and concluding remarks

We have witnessed important insights and discoveries in the physiology and pathophysiology of BA in the last decades, which, of course, include different cellular and molecular aspects. Slowly, one starts to appreciate the complex network of interactions that form the basis of these processes. Obviously, the development of new and more sensitive analytical tools was an important milestone and enabled breakthroughs in all areas of BA research. One has also to stress that such methodological improvements are also required in the future to drive research to the next level. Despite all of these discoveries, we only start to see the tip of the iceberg, and the future will for sure hold many surprises in our understanding of function, regulation, and pathophysiology of bile acids.

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