Bile Acids and TGR5 (Gpbar1) Signaling

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Bile Acid Receptors and Bile Acid Sensing Molecules

Bile acid (BA) effects are mediated through different types of BA receptors and sensing molecules, which allow for a cell type- and BA-specific signaling (Fig. 1) [1–5]. Nuclear BA receptors are ligand activated transcription factors and comprise the farnesoid X receptor (FXR, NR1H4) [6–11], the pregnane X receptor (PXR, NR1I2) [12, 13], and the vitamin D receptor (VDR, NR1I1) [14–16]. FXR is the master regulator of BA homeostasis and is activated by the primary BA chenodeoxycholic acid (CDCA) and its conjugates with an EC_{50} of approximately 5–20 μM [6, 8, 10, 11, 17, 18]. The secondary BAs deoxycholic acid (DCA) and lithocholic acid (LCA) are also FXR ligands, however less efficient than CDCA [8, 10, 17, 19]. In contrast, only the secondary BA LCA acts as ligand for PXR and VDR [12, 13, 16].

Besides activation of intracellular nuclear receptors, BAs can modulate the signaling of several G protein-coupled receptors (GPCRs) at the cell surface, such as different types of muscarinic (acetylcholine) receptors (e.g., M2 and M3 receptors) [20–23] as well as formyl peptide receptors (FPR) [5, 24, 25]. Furthermore, taurine-conjugated BAs are ligands for the sphingosine-1-phosphate receptor 2 (S1PR2), which is expressed in liver parenchymal cells (hepatocytes) where it regulates sterol and lipid metabolism as well as in cholangiocytes, where its activation triggers cell proliferation [26–31]. TGR5 (Gpbar1, M-BAR) is a GPCR that predominately couples to a stimulatory G protein and is activated by both conjugated and unconjugated primary and secondary BAs [32–34].

Integrins (α5β1) also serve as BA sensing molecules in hepatocytes for taurine-conjugated ursodeoxycholic acid (TUDCA) [35–37]. Uptake of BAs across the plasma membrane is a prerequisite for BA-mediated α5β1 integrin activation since

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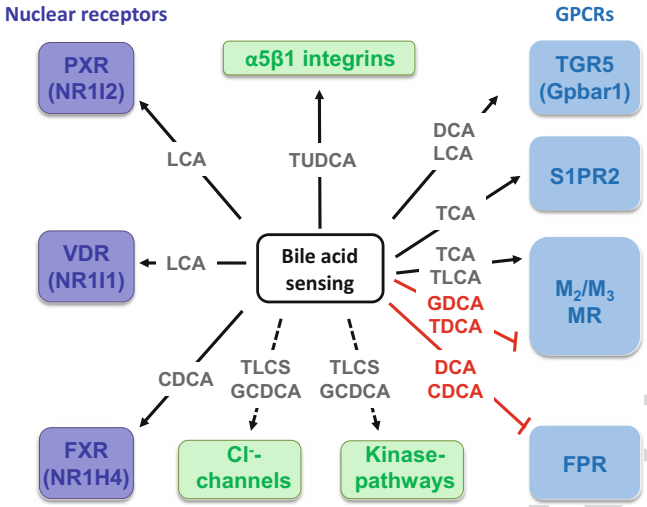

Fig. 1 Bile acid sensing molecules. Several BA responsive receptors and molecules have been identified. Three nuclear receptors (NR) have been demonstrated to be activated by BAs: the pregnane X receptor (PXR), the vitamin D receptor (VDR) and the farnesoid X receptor (FXR) (purple boxes). Moreover, multiple G protein-coupled receptors (GPCRs) are either directly activated or modulated in their activity by different BAs (blue boxes). While TGR5 (Gpbar-1) and the sphingosine-1-phosphate receptor 2 (S1PR2) are activated by various BAs, other GPCRs, such as the formyl peptide receptor (FPR) and the muscarinic acetylcholine receptors M_2 and M_3 can be inhibited in their signaling by BA. Furthermore, α5β1-integrins, chloride channels, and several kinase pathways are activated by various bile acids (green boxes). Dashed arrows indicate potentially indirect signaling, black arrows indicate a stimulatory effect, while inhibitory effects are depicted in red. CDCA chenodeoxycholic acid, GCDCA glycochenodeoxycholic acid, TCA taurocholic acid, TLCA taurolithocholic acid, TLCS taurolithocholylsulfate, LCA lithocholic acid, DCA deoxycholic acid, TUDCA tauroursodeoxycholic acid. Modified after [1]

α5β1 is found on intracellular endomembranes. Activation of α5β1 by TUDCA results in increased bile secretion (choleresis), cell proliferation, and also protects the cells from death receptor-mediated apoptosis [35–37].

Further BA sensors include ion channels and kinase signaling pathways; however, the precise mechanism by which BAs modulate these signaling molecules remains elusive [1, 38–43].

The presence of various nuclear and plasma membrane-bound receptors for BAs not only allow for a cell type- and BA-specific signaling but also help to explain the pleiotropic effects of BAs in the organism.

TGR5, a G Protein-Coupled Receptor for Bile Acids

TGR5 was discovered and characterized as a G protein-coupled receptor for both primary and secondary BAs by Maruyama et al. in 2002 and Kawamata et al. in 2003 [32, 33].

The gene encoding human TGR5 is located in the chromosomal region 2q35 and consists of two exons [44]. The coding region is entirely located in exon 2, encompasses 993 base pairs (bp), and translates into 330 amino acids [33, 44]. The coding regions of rat and mouse Tgr5 contain 990 bp and encode for 329 amino acids each. There is a high sequence conservation between human, bovine, rabbit, rat, and mouse TGR5 with amino acid identities ranging from 82 to 91% [32, 33]. TGR5 belongs to the class A of GPCRs (rhodopsin-like GPCRs) and shows the highest amino acid identity to different sphingosine-1-phosphate receptors (S1PR) [32, 33], which is below 30%, however.

TGR5-Dependent Intracellular Signaling Pathways

Heterotrimeric G proteins are formed from an α-subunit, which binds and hydrolyses guanosine triphosphate (GTP), and a complex of a β- and a γ-subunit [45–47]. Four different classes of α-subunits are distinguished: $G\alpha_s$ promotes activation of adenylate cyclase, $G\alpha_{i/o}$ leads to inhibition of adenylate cyclase, $G\alpha_{q/11}$ triggers activation of phospholipase Cβ, and $G\alpha_{12/13}$ are associated with stimulation of Rho guanine-nucleotide exchange factors (GEFs) [45–47]. BA binding to TGR5 leads to an activation of the receptor and association with a G protein consisting of the GDP-bound α-subunit and a βγ-complex [45]. Following the interaction of the GPCR with the G protein, GDP is released and replaced by GTP, which in turn triggers a conformational change of the α-subunit and the subsequent dissociation of the α-subunit from the βγ-complex [45]. Further downstream signaling is then initiated by the α-subunit and the βγ-complex, respectively [45]. In most cell types studied to date, TGR5 will associate with a $G\alpha_s/\beta\gamma$ heterotrimer and thus trigger the activation of adenylate cyclase resulting in an elevation of intracellular cyclic AMP (cAMP) levels [32, 33]. Downstream signaling activated by TGR5 comprise protein kinase A (PKA)-, protein kinase B (AKT)-, mammalian target of rapamycin complex 1 (mTORC1)- and extracellular-signal regulated kinase (ERK)-pathways [32, 48–52]. Furthermore, stimulation of TGR5 results in inhibition of nuclear factor kappa B (NFκB) signaling, elevation of intracellular calcium levels, and activation of different ion channels and modification of gene expression [48, 50, 53–60].

Similar to the S1PR2, TGR5 can couple to different G proteins [27, 32, 33, 49, 61]. It was demonstrated that the receptor can couple to both $G\alpha_s$ as well as to an inhibitory G alpha protein ($G\alpha_i$) in biliary epithelial cells depending on the subcellular localization of TGR5 [49]. TGR5 located in the primary cilia of cholangiocytes coupled to $G\alpha_i$ and attenuated cell proliferation, while TGR5 located on the apical plasma membrane associated with a $G\alpha_s$ protein upon ligand binding and triggered cell proliferation [49]. In the FLO cell line, which is derived from human esophageal Barrett's adenocarcinoma, co-immunoprecipitation experiments demonstrated that TGR5 could interact with both $G\alpha_q$ and $G\alpha_{i3}$; however, signal transduction after ligand binding was mediated only by $G\alpha_q$ [61]. Thus, cell type and subcellular localization seem to determine the interaction of TGR5 with a specific G alpha

protein. Whether posttranslational modifications also contribute to the G alpha protein subclass selectivity of TGR5 is unknown.

TGR5 Ligand Binding and Selectivity

TGR5 recognizes a wide spectrum of ligands, ranging from BAs and neurosteroids as natural TGR5 agonists to synthetic BAs and agonists with a nonsteroidal core (Fig. 2) [34, 62]. Particularly, several nonsteroidal intestine-specific TGR5 agonists are known [63, 64]. The specificity is achieved by the presence of quaternary ammonium groups or by a considerable ligand size; for the latter, two TGR5 agonists are coupled via a linker region (**15c** in Fig. 2). In contrast to other BA receptors, TGR5 is activated by all known BAs, regardless of their substitution pattern and state of conjugation (un-, taurine- or glycine-conjugated), although with varying levels of potency ranging from 0.29 to 36.7 μM [34]. Generally, the agonistic potential of BAs toward TGR5 increases with the hydrophobicity of the cholane scaffold. The most potent natural agonist of TGR5 with an EC_{50} of 0.29 μM is the secondary BA taurolithocholic acid (TLCA), which is hydroxylated in position 3 of the cholane scaffold only (Fig. 2). Additional hydroxylation of position 12 in the secondary BA deoxycholic acid (DCA) or position 7 in the primary BA chenodeoxycholic acid (CDCA) increases the EC_{50} 4-fold and 23-fold compared to TLCA, respectively (Fig. 2). The stereochemical configuration of the hydroxyl group in position 7 of the cholane scaffold has a large impact on TGR5 activation: The epimers CDCA and ursodeoxycholic acid (UDCA) show a fivefold difference in their efficacy as TGR5 agonists, with CDCA being more potent. This epimeric selectivity has been explained by a hydrogen bond formation of CDCA's 7-α-hydroxyl group to Y89 in transmembrane helix 3 (TM3) of TGR5 (Fig. 3a) [65]. In contrast, due to the β-configuration, UDCA cannot form such a hydrogen bond with its 7-hydroxyl group (Fig. 3b).

The BAs' conjugation is another factor influencing their efficacy toward TGR5. BAs with a free acid moiety and the respective glycine-conjugated derivatives generally exhibit a similar potency, as seen in lithocholic acid (LCA; EC_{50} 0.58 μM) and glycolithocholic acid (GLCA; EC_{50} 0.54 μM). However, taurine-conjugated derivatives are more potent than their related BAs, e.g., TLCA with an EC_{50} of 0.29 μM compared to LCA. Taurine conjugation increases the size of a BA more than glycine conjugation, which allows the bridging of the residues R79 (EL1) and Y240 (TM 6) in TGR5 (Fig. 3c). The salt-bridge interaction between the negatively charged sulfonic acid moiety and the positively charged R79 likely increases the affinity of those BAs toward TGR5 [65]. All BAs employ the 3-hydroxyl 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) (Fig. 3b). The interaction with Y240 is crucial for the activation of TGR5, as mutation of this residue to alanine or phenylalanine abrogates TGR5 signaling [65]. Agonistic neurosteroids such as pregnanediol (Fig. 2) also utilize their hydroxyl or carbonyl groups to interact with Y240 in TGR5. Lacking acidic groups, they mainly form

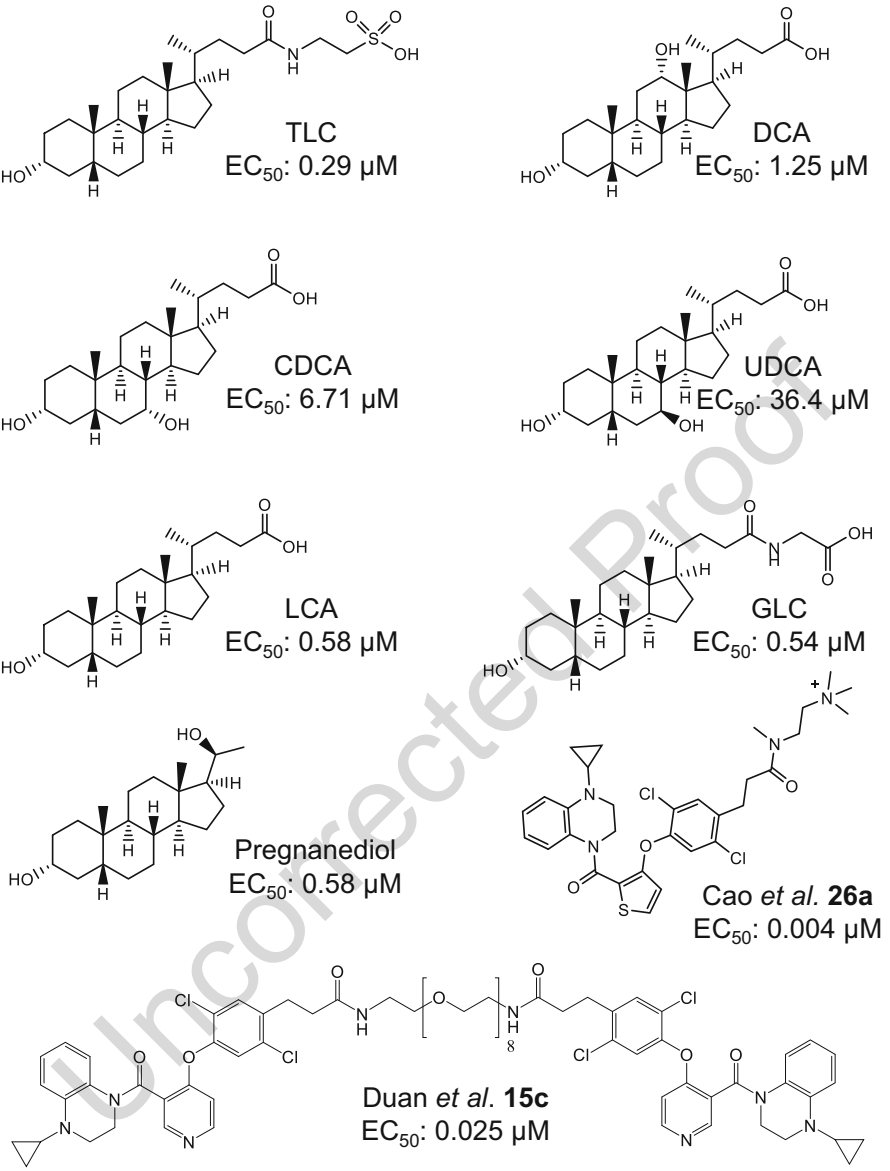

Fig. 2 Bile acid agonists and their EC_{50} values toward TGR5 as reported in Ref. [34, 63]. Primary bile acids: CDCA and UDCA. Secondary bile acids: DCA, LCA, GLC, and TLC. Intestine-specific nonsteroidal TGR5-specific agonists **26a** from Ref. [63] and **15c** from Ref. [64]. The primary bile acids are generally less effective TGR5 agonists than the secondary bile acids. The configuration of the hydroxyl group in position seven (if present) strongly influences the activity: The α-configuration as present in CDCA is more favorable than the β-configuration in UDCA. Conjugation of the acid moiety with glycine increases the activity toward TGR5 only slightly, while taurine conjugation increases the activity markedly

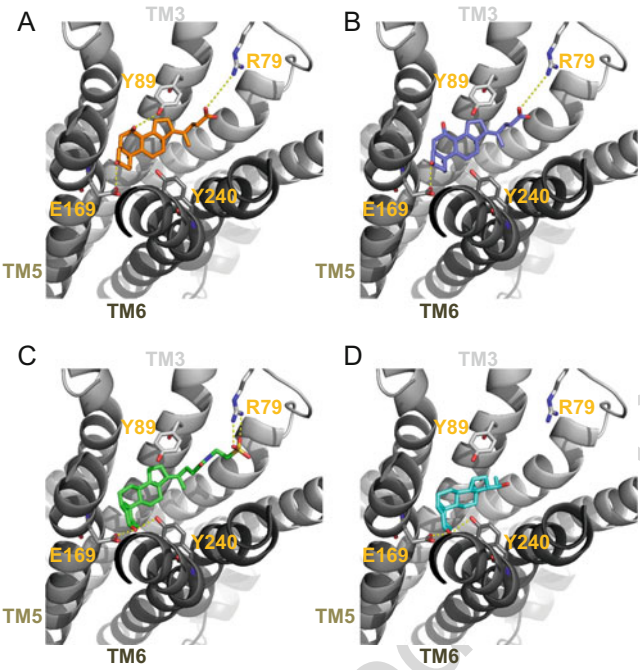

Fig. 3 Binding modes of bile acids and neurosteroids as reported in Ref. [65]. (**a**) Binding mode of CDCA in TGR5. CDCA forms a hydrogen bond to E169 in TM5 (yellow dotted line) and a weak hydrogen bond to Y240 in TM6. Additionally, the 7α-hydroxyl group of CDCA forms a hydrogen bond to Y89 in TM3 (yellow dotted line), and a salt bridge with R79 (yellow dotted line). (**b**) Binding mode of UDCA in TGR5. UDCA forms a hydrogen bond to E169 in TM5 (yellow dotted line) and a weak hydrogen bond to Y240 in TM6. Unlike CDCA, UDCA is unable to form a hydrogen bond to Y89 due to the β-configuration of its 7-hydroxyl group, resulting in a lower efficacy compared to CDCA. (**c**) Binding mode of TLC in TGR5. TLC forms hydrogen bonds to E169 in TM5 and to Y240 in TM6 (yellow dotted lines). With its sulfonic acid moiety, it forms a salt bridge to R79 (yellow dotted lines). These interactions may explain why TLC is the most potent natural bile acid toward TGR5. (**d**) Binding mode of pregnanediol in TGR5. Pregnanediol forms hydrogen bonds to E169 in TM5 and to Y240 in TM6 (yellow dotted line). Lacking an acid group, it mainly forms hydrophobic contacts with Y89 in TM3

126 additional hydrophobic contacts with Y89 in TM3 to bind to and activate TGR5
127 (Fig. 3d) at a reasonable EC_{50} [e.g., pregnanediol (Figs. 2 and 3d), EC_{50} 0.58 μM],
128 allowing them to activate TGR5 in the brain [34, 54].

129 TGR5-specific agonists with a nonsteroidal core mimic BAs through the presence
130 of an acid or amide moiety, which is linked to a system of three to four variably
131 interconnected aromatic and aliphatic rings. The ring furthest from the acid or amide
132 moiety always contains a heteroatom (e.g., **26a**, **15c** in Fig. 2). Although the binding
133 mode of nonsteroidal TGR5 agonists is unknown, it is possible that the heteroatom is
134 necessary to form a hydrogen bond to Y240 (TM6), which is crucial for the
135 activation of TGR5. Finally, as TGR5 binds ligands of various shapes and sizes, it
136 is surprising that to date no antagonist of TGR5 is known. All the more because it is

often easier to develop ligands that bind to GPCRs but do not activate them, as such ligands do not need to bridge TMs 3 and 6 in a specific manner to induce the movement of TM6 leading to GPCR activation [66].

TGR5 Tissue Distribution

TGR5 mRNA was detected almost ubiquitously in human and rodent tissues [32, 67, 68]. In mice, the strongest signal for TGR5 expression was detected in the gallbladder, followed by high expression levels in the spleen, lung, placenta as well as ileum and colon [67, 68]. In human tissues, a similar expression pattern was found with high TGR5 mRNA levels in gallbladder, placenta, spleen, lung, liver, stomach, small intestine, uterus, and mammary gland [32, 53, 69]. On the protein level, TGR5 has been detected in CD14-positive monocytes and tissue-resident macrophages in both humans and rodents, in different nonparenchymal cells of the liver, in gallbladder epithelial cells and gallbladder smooth muscle cells, in astrocytes, neurons and microglia in the central as well as in astrocytes and neurons of the enteric and peripheral nervous system [4, 48–50, 53–58, 70–73]. Furthermore, TGR5 has been localized in intestinal epithelial cells, enteroendocrine L-cells, in human kidney proximal tubule cells and podocytes, in murine brown adipocytes, in human skeletal muscle cells and in pancreatic β cells [56, 59, 74–79].

In rodent and human liver, TGR5 is localized in sinusoidal endothelial cells (LSEC), in liver resident macrophages (Kupffer cells, KC), and in cholangiocytes [4, 52, 57, 58, 71, 80]. While quiescent hepatic stellate cells (HSC) do not express TGR5, the receptor is upregulated during culture of isolated HSC and can also be detected in activated, myofibroblast-like HSC in vivo [57, 81]. Using immunofluorescence staining of rat and human liver cryosection, TGR5 has not been detected in hepatocytes, indicating that expression levels are much lower as compared to the TGR5-expressing nonparenchymal liver cells [52, 57].

Regulation of TGR5 Expression, Localization, and Function

Very little is known on the regulation of TGR5 expression, localization, and function to date. An upregulation of TGR5 mRNA has been observed in the frontal cortex of mice following acute liver failure, which was induced by intraperitoneal injection of azoxymethane [82]. In contrast, a downregulation of the receptor has been demonstrated in isolated rat astrocytes following stimulation with ammonia (NH_4Cl; 0.5–5 mM, 72 h) both on the mRNA and protein level. In line with this finding, reduced levels of TGR5 mRNA were detected in cortical brain tissue from patients with hepatic encephalopathy as compared to samples from control subjects [54].

Stimulation of either rat astrocytes or human macrophages with the TGR5 agonistic progesterone metabolites 5β-pregnan-3α-ol-20-one or 5α-pregnan-

3α-ol-20-one and 5α-pregnan-3β-ol-20-one triggered a significant downregulation of TGR5 mRNA levels [54, 69].

Thus, downregulation of TGR5 mRNA expression may represent a mechanism of receptor desensitization in response to continuous stimulation [54, 69]. This may be highly relevant since TGR5 unlike many other GPCRs does not interact with β-arrestins 1 and 2 or G protein-coupled receptor kinases 2, 5, or 6 and therefore does not traffic from the plasma membrane to endosomes in response to activation [83]. Ligand binding to TGR5 in the plasma membrane induced a sustained cAMP response, indicating that TGR5 does not desensitize to repetitive stimulation [83].

TGR5 Functions in Liver in Health and Disease

Role of TGR5 for Bile Acid Homeostasis and Bile Secretion Under Physiological and Cholestatic Conditions

Targeted deletion of TGR5 in mice is not associated with an obvious phenotype or the spontaneous development of liver disease [67, 68]. However, TGR5 knockout mice have a smaller BA pool size, despite unchanged expression levels of the rate-limiting enzyme of BA synthesis Cyp7a1 and similar fecal excretion rates of BAs as wild-type littermates [4, 5, 67, 72]. Bile acid pool composition is also altered in absence of TGR5 with a relative increase in taurocholic acid (TCA) and taurodeoxycholic acid (TDCA) and a decrease of tauro-β-muricholic acid (TβMCA), which may be attributed to lower Cyp7b1 expression [72, 84].

In cholangiocytes and gallbladder epithelial cells, TGR5 is localized in the primary cilia, which extend from the plasma membrane into the bile duct or gallbladder lumen, as well as on the apical plasma membrane [49, 53, 71]. Ligand binding to TGR5 on biliary epithelial cells triggers elevation of intracellular cAMP, which in turn promotes CFTR (ABCC7)-dependent chloride secretion [53, 80, 85]. Subsequently, chloride is exchanged across the apical plasma membrane against bicarbonate by the anion exchanger 2 (AE2, SLC4A2), thereby promoting formation of a protective bicarbonate film/bicarbonate umbrella as well as bicarbonate-rich biliary bile flow (choleresis) [53, 72, 80, 85–89]. Since not only transport activity but also surface expression of CFTR and AE2 is regulated by cAMP, stimulation of TGR5 increases chloride and bicarbonate secretion directly and also indirectly through enhanced insertion of CFTR and AE2 into the apical plasma membrane from intracellular vesicles [53, 80, 85]. The bicarbonate umbrella together with the glycocalix creates an alkaline microenvironment, which hampers the protonation of hydrophobic glycine-conjugated BAs, inhibits diffusion of protonated apolar BAs across the apical membrane of biliary epithelial cells and thus protects the cells from BA toxicity [85, 87–90]. Therefore, it is not surprising that cholangiocytes from TGR5 knockout mice are more susceptible toward BA-induced cell damage [52]. Besides maintenance of the bicarbonate umbrella, TGR5 exerts antiapoptotic effects in biliary epithelial cells via serine phosphorylation of the CD95 death receptor [52]. Activation of TGR5 also triggers cholangiocyte proliferation through

elevation of reactive oxygen species, subsequent activation of Src kinase, matrix-metalloproteinase-dependent shedding of epidermal growth factor (EGF), transactivation of the epidermal growth factor receptor (EGFR), and subsequent phosphorylation of mitogen-activated kinases (MAPK) ERK1/2 [52]. Cholangiocyte proliferation in response to BA feeding (CA, LCA) or common bile duct ligation (CBDL) is impaired in TGR5 knockout mice in vivo [52]. Besides a reduced cholangiocyte proliferative response, TGR5 knockout mice are more susceptible toward bile acid-mediated, cholestatic liver injury [52, 91, 92]. Cholic acid (0.5% for 7 days or 1% for 5 days) feeding and CBDL for up to 7 days resulted in a more pronounced liver injury in the absence of TGR5 as demonstrated by higher levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) and/or more pronounced liver cell necrosis on histology [52, 91, 92]. Livers from TGR5 knockout mice not only displayed significantly decreased cholangiocyte but also significantly reduced hepatocyte proliferation [52]. The mechanisms underlying reduced hepatocyte proliferation in TGR5 knockout mice remain elusive to date, since TGR5 protein levels are below the detection level in hepatocytes [57]. The impaired proliferative response of hepatocytes in mice with targeted deletion of TGR5 has also been observed after partial hepatectomy (PHx) [91]. Following PHx, concentrations of hepatic BAs were elevated and biliary BA composition was more hydrophobic in the absence of TGR5 [91]. Treatment with the BA binding resin cholestyramine (2%) alleviated liver injury in TGR5 knockout mice, suggesting that the higher hepatic BA levels as well as the altered composition of the BA pool contribute to the observed phenotype [91, 92].

Gallbladder volume was decreased in TGR5 knockout mice as compared to wild-type animals both on chow as well as on BA (CA, 0.2%)-enriched diet [48, 72, 92], which was attributed to reduced TGR5-dependent biliary secretion but also to impaired smooth muscle cell relaxation in the absence of TGR5 [48, 72]. In contrast, gallbladder size of wild-type mice increased up to 230% following administration of different synthetic TGR5 agonists (6α-ethyl-23(S)-methyl-cholic acid (INT-777), a 4-phenoxypyrimidine-5-carboxamide derivative (compound 18) or a 4-phenoxynicotinamide derivative (compound 23 g)) [72, 93, 94]. Although gallbladder hypomotility, as observed in TGR5 knockout mice, is associated with increased risk of cholesterol gallstone formation [72, 95], mice with targeted deletion of TGR5 did not develop cholesterol gallstones when fed a lithogenic diet [68].

Immunomodulatory and Metabolic Functions of TGR5 in Liver

In macrophages, stimulation of TGR5 suppresses inflammatory cytokine and chemokine expression and secretion, inhibits phagocytosis and migration and induces an anti-inflammatory macrophage phenotype, characterized by maintained expression of interleukin (IL) 10 despite downregulation of pro-inflammatory cytokines [50, 55, 57, 96–99]. The mechanism underlying reduced inflammatory cytokine expression comprises TGR5-dependent elevation of cAMP and subsequent inhibition of I kappa B kinase (IKK), which in turn prevents phosphorylation of the

inhibitor of nuclear factor-κB (IκB) and thus hampers the nuclear translocation of NF-κB-p65 resulting in reduced transcriptional activity of NF-κB [55, 100]. The signaling pathway resulting in decreased chemokine secretion is dependent on an AKT-mediated activation of the mTOR complex-1 (mTORC-1), which increases the relative expression and protein levels of the dominant-negative CCAAT/enhancer binding protein β (C/EBPβ) isoform liver inhibitory protein (LIP) thereby suppressing expression of chemokines such as Ccl2, Ccl3, and Ccl4 [50, 100].

In vivo, intraperitoneal injection of lipopolysaccharide (LPS) resulted in a more severe phenotype as well as liver injury in TGR5 knockout mice as compared to wild-type animals, which was characterized by significantly increased mortality (Reich, Häussinger, Keitel unpublished), elevated levels for alanine (ALT) and aspartate (AST) aminotransferases, enhanced inflammatory infiltrates in liver tissue, and increased hepatocyte apoptosis [97]. TGR5 knockout mice were also more susceptible to infection with *Listeria monocytogenes* (8×10^4 CFU/ml) as demonstrated by a significantly higher mortality rate, increased listeria titers in liver and spleen as well as a more aggravated liver inflammation and damage (Reich, Häussinger, Keitel, unpublished) (Fig. 4). AU2

Activation of TGR5 has been shown not only to exert anti-inflammatory effects in liver and adipose tissue but also to improve various aspects of the metabolic syndrome, such as obesity, insulin resistance, and atherosclerosis [5, 55, 56, 59]. Treatment of wild-type mice fed a high fat diet (HFD) with the TGR5 agonist INT-777 attenuated obesity, reduced fat mass, and improved glucose tolerance through increased intestinal glucagon-like peptide-1 (GLP-1) secretion [56]. Furthermore, administration of INT-777 lowered liver fatty acid and triglyceride concentrations resulting in decreased hepatic steatosis and improved serum ALT and AST levels as compared to the HFD-fed control animals [56]. The beneficial effects of TGR5 agonist on steatohepatitis may be attributed to reduced hepatic and adipose tissue inflammation, to an increase in TGR5-mediated energy expenditure and an improved insulin sensitivity due to enhanced intestinal GLP-1 secretion [50, 56, 59, 100]. Whether direct effects of TGR5 agonists on hepatocytes also contribute to the attenuation or improvement of steatohepatitis in mice on HFD or obese mice remains unknown. Treatment of obese db/db mice with a dual FXR/TGR5 agonist (6α-ethyl-24-nor-5β-cholane-3α,7α,23-trio-23-sulfate sodium salt, INT-767) for 6 weeks increased the proportion of intrahepatic $Ly6C^{low}$ anti-inflammatory macrophages and ameliorated steatohepatitis as assessed by histology [101]. This is in line with results from human macrophages, where bile acid treatment in a TGR5-dependent way promoted the differentiation of an anti-inflammatory macrophage phenotype, characterized by an increased IL10/IL12 ratio [96].

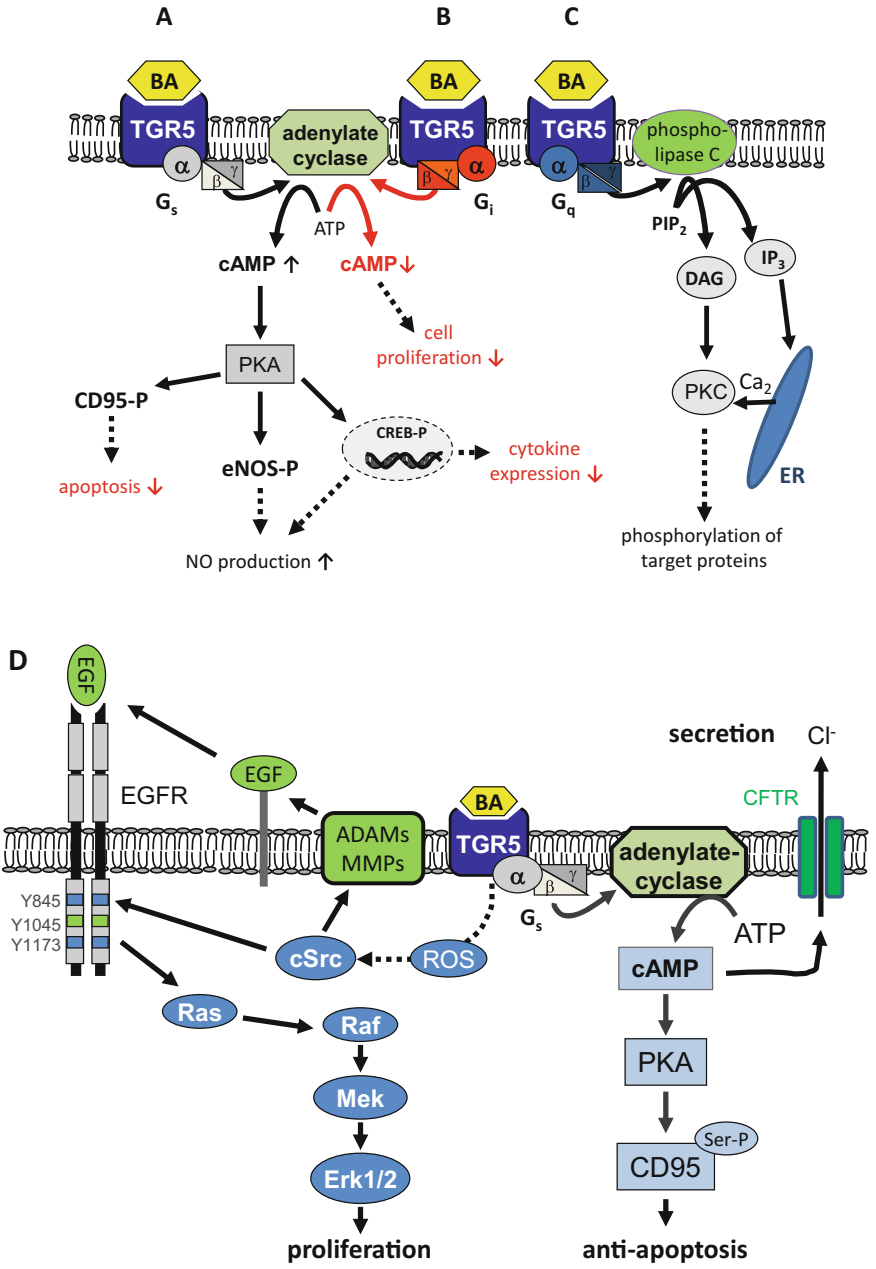

Fig. 4 TGR5-dependent bile acid signaling. (**a**) In most cell types TGR5 is associated with a G_S-protein, therefore, ligand binding triggers and activation of adenylate cyclase inducing an increase of the ATP-dependent cAMP production and an activation of the protein kinase A (PKA), which in turn may trigger several different signaling pathways. (**b**) Furthermore, interaction of TGR5 with G_i-proteins inhibiting adenylate cyclase activity has been demonstrated for ciliated

Role of TGR5 in Sinusoidal Endothelial Cells and Hepatic Stellate Cells

Liver sinusoidal endothelial cells (LSEC) are exposed to varying concentrations of nutrients, including BAs. After food intake, BA levels rise in portal venous blood and reach concentrations between 14 and 43 μM [1, 102–104]. Ligand binding to TGR5 on LSEC triggered not only increased expression of endothelial NO synthase (eNOS) but also stimulated phosphorylation of eNOS at serine 1177 via activation of protein kinase A (PKA), resulting in increased NO production in rat liver slices [58]. Similar results were obtained after TLCA treatment of endothelial cells from bovine aorta or human umbilical vein [105]. Furthermore, TLCA inhibited LPS-mediated upregulation of vascular cell adhesion molecule-1 (VCAM-1) and subsequent monocyte [105]. LSEC are an important NO donor in the hepatic sinusoids. Decreased NO production in LSEC is one hallmark of portal hypertension [1, 106–109]. Thus, stimulation of TGR5 and activation of the cAMP-PKA-eNOS-NO downstream signaling pathway may be beneficial in portal hypertension [1, 4]. In carbon tetrachloride treated mice simultaneous administration of a TGR5 agonist (6β-ethyl-3α,7β-dihydroxy-5β-cholan-24-ol (BAR501) 15 mg/kg/day) did not protect the animals from development of liver fibrosis, however inhibited the development of endothelial dysfunction and portal hypertension [110]. This beneficial effect was associated with reduced expression of endothelin-1 and increased expression of cystathionine-y-lyase (CSE), an enzyme responsible for the generation of the vasodilatory agent hydrogen sulfide [110].

TGR5 mRNA expression was below the detection level in freshly isolated HSC but increased significantly within days in culture [57, 81]. In activated, myofibroblast-like HSC elevation of cAMP led to an internalization of the endothelin-A (ET-A) receptor thereby attenuating the contractile response of the cells toward endothelin-1 [111]. Stimulation of TGR5 on activated HSC may trigger cAMP-mediated ET-A receptor desensitization and thereby contribute to reduced portal pressure.

While activation of the TGR5-cAMP-PKA-eNOS-NO signaling pathway in LSEC may allow for adaption of sinusoidal blood flow in response to nutrient intake thereby promoting hepatic metabolism under physiological conditions, stimulation of TGR5 on LSEC and activated HSC may attenuate portal hypertension development after liver damage [1].

Fig. 4 (continued) cholangiocytes, where activation of TGR5 inhibited cell proliferation [49]. (**c**) Coupling of TGR5 with G_q-proteins has been observed in the oesophageal adenocarcinoma cell line (FLO) and triggered expression of NADPH oxidase NOX-5 and cell proliferation [61]. G_q-proteins may signal through activation of phospholipase C which in turn triggers the synthesis of diacylglyerol (DAG) and inositol trisphosphate (IP_3). By induction of proteinkinase C (PKC) activity and release of intracellular Ca_2 from the endoplasmatic reticulum (ER) these two second messenger proteins are causing the phosphorylation of different target proteins. (**d**) TGR5-dependent signaling in cholangiocytes. Modified after [52]

TGR5 in Human Liver Disease

In contrast to mice little is known on the role of TGR5 for the pathogenesis of human liver diseases [92]. In line with the high expression of TGR5 in cholangiocytes, TGR5 expression, localization, and function have been studied in biliary diseases. TGR5 protein levels as measured by relative quantification of TGR5 immunofluorescence staining in relation to cytokeratin 7 staining were significantly higher in human cholangiocarcinoma (CCA) tissue as compared to cholangiocytes from the nontumorous resection margins [52, 85]. Using CCA-derived cell lines (EGI-1 and TFK-1), it was demonstrated that activation of TGR5 triggers cell proliferation using the same ROS-cSrc-MMP-EGFR-ERK1/2 signaling pathway as in cultured murine cholangiocytes [52]. Furthermore, TGR5 stimulation induced apoptosis resistance and promoted cell migration and invasiveness. Thus, the receptor may contribute to CCA progression.

An overexpression of TGR5 has also been described in cystic cholangiocytes of polycystic liver disease (PLD) [112]. Stimulation of TGR5 in rodent cystic cholangiocytes promotes a rise in intracellular cAMP, which triggers proliferation and cyst growth, while deletion of TGR5 in a rodent model of PLD attenuates cyst formation [112].

In contrast to CCA- and PLD-derived biliary cells, which are characterized by high TGR5 expression levels, a reduction in TGR5 immunofluorescence staining intensity has been observed in cholangiocytes of livers from patients with primary sclerosing cholangitis (PSC) as well as in livers from Abcb4 (Mdr2) knockout mice, which serve as an animal model for PSC [52, 85, 92, 112]. The mechanisms as well as the timing (early or late) of the TGR5 downregulation in the disease course of PSC is yet unclear [85, 92]; however, the reduced TGR5 expression may render cholangiocytes more susceptible toward BA-mediated cytotoxicity and thus accelerate disease progression [92].

Conclusion

Bile acids are signaling molecules with pleiotropic endocrine and paracrine functions which are mediated by multiple BA sensing molecules, thus enabling a BA- and cell type-specific response. BAs regulate bile acid, glucose, lipid and energy homeostasis, modulate the immune response and affect cell survival and cell proliferation. Therefore, BAs and BA sensors have emerged as attractive targets for the treatment of metabolic diseases such as steatohepatitis, obesity, diabetes, and atherosclerosis. TGR5 (Gpbar1, M-Bar) is a G protein-coupled receptor highly responsive to primary and secondary bile acids as well as to various progesterone metabolites. The receptor is almost ubiquitously expressed and has been detected in tissues participating in bile acid synthesis and secretion such as liver, intestine, and kidney. However, TGR5 is also found in placenta, adrenal glands, and brain, where the receptor may primarily serve as membrane-bound receptor for steroid hormones. In line with the broad tissue expression TGR5 has numerous functions including

modulation of the immune response, regulation of glucose and energy homeostasis as well as intestinal motility. In liver, TGR5 activation can modulate liver microcirculation, promote biliary secretion and proliferation of biliary epithelial cells, induce gallbladder filling and exert anti-inflammatory effects. Targeted deletion of TGR5 renders mice more susceptible toward inflammatory as well as cholestatic liver injury and impairs liver regeneration. In contrast, pharmacological stimulation of TGR5 improves steatohepatitis.

While TGR5 is overexpressed in cholangiocarcinoma tissue and promotes apoptosis resistance, cell proliferation, cell migration and invasiveness in CCA cell lines, the receptor is downregulated in cholangiocytes of livers from patients with progressive sclerosing cholangitis (PSC) as well as in livers from Mdr2 knockout mice, which serve as an animal model for PSC. Further studies are needed to elucidate the role of TGR5 in human liver disease.

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AU2	Please check and confirm that the inserted citation of Fig. 4 is appropriate here.	
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AU4	Please check and confirm the edit made in the sentence beginning "Stimulation of TGR5 on activated HSC may trigger..."	