Targeting FXR in Cholestasis

Verena Keitel, Carola Dröge, and Dieter Häussinger

Contents

Abstract

The farnesoid X receptor (FXR, NR1H4) is a bile acid (BA)-activated transcription factor, which is essential for BA homeostasis. FXR and its hepatic and intestinal target genes, small heterodimer partner (SHP, NR0B2) and fibroblast growth factor 15/19 (Fgf15 in mice, FGF19 in humans), transcriptionally regulate BA synthesis, detoxification, secretion, and absorption in the enterohepatic circulation. Furthermore, FXR modulates a large variety of physiological processes, such as lipid and glucose homeostasis as well as the inflammatory response. Targeted deletion of FXR renders mice highly susceptible to cholic acid feeding resulting in cholestatic liver injury, weight loss, and increased mortality. Combined deletion of FXR and SHP spontaneously triggers early-onset intrahepatic

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cholestasis in mice resembling human progressive familial intrahepatic cholestasis (PFIC). Reduced expression levels and activity of FXR have been reported in human cholestatic conditions, such as PFIC type 1 and intrahepatic cholestasis of pregnancy. Recently, two pairs of siblings with homozygous FXR truncation or deletion variants were identified. All four children suffered from severe, early-onset PFIC and liver failure leading to death or need for liver transplantation before the age of 2. These findings underscore the central role of FXR as regulator of systemic and hepatic BA levels. Therefore, targeting FXR has been exploited in different animal models of both intrahepatic and obstructive cholestasis, and the first FXR agonist obeticholic acid (OCA) has been approved for the treatment of primary biliary cholangitis (PBC). Further FXR agonists as well as a FGF19 analogue are currently tested in clinical trials for different cholestatic liver diseases. This chapter will summarize the current knowledge on the role of FXR in cholestasis both in rodent models and in human diseases.

Keywords

Bile acid homeostasis · Cholestasis · Farnesoid X receptor (FXR) · FGF19 · Fibroblast growth factor · Obeticholic acid · Primary biliary cholangitis (PBC) · Primary sclerosing cholangitis (PSC)

1 Introduction

Cholestasis describes the impaired formation and/or secretion of bile into the small intestine. Clinically, cholestasis can be classified into intrahepatic or extrahepatic as well as into obstructive or nonobstructive. Obstructive cholestasis can affect the intrahepatic and the extrahepatic biliary tree or both. Common causes of biliary obstruction are malignancies, gallstones, cysts, or strictures, the latter including biliary atresia (European Association for the Study of the Liver 2009). Intrahepatic cholestasis may result from defective BA synthesis, from impaired secretory functions of hepatocytes and/or cholangiocytes, or from obstruction of the intrahepatic biliary tree distal of the canal of Hering (European Association for the Study of the Liver 2009). Hepatocytic bile formation comprises the synthesis of BAs from cholesterol as well as the secretion of conjugated BAs across the canalicular membrane and is controlled by FXR signaling.

FXR belongs to the superfamily of nuclear receptors (NRs), which act as ligand-activated transcription factors (Forman et al. 1995; Makishima et al. 1999; Parks et al. 1999; Wang et al. 1999). Human primary and secondary BAs such as chenodeoxycholic acid (CDCA), deoxycholic acid (DCA), lithocholic acid (LCA), and their taurine and glycine conjugates serve as ligands for FXR (Makishima et al. 1999; Parks et al. 1999; Wang et al. 1999). The role of FXR as a master regulator of BA homeostasis is underscored by the high expression of the receptor in the liver, ileum, as well as kidney and by dysregulation of BA homeostasis in FXR knockout mice (Forman et al. 1995; Sinal et al. 2000).

In hepatocytes, BAs suppress the transcription of the rate-controlling enzyme in BA synthesis, CYP7A1 as well as CYP8B1, which is the enzyme controlling cholic

acid (CA) production (Russell 2003). One of the FXR downstream targets is the small heterodimer partner (SHP, NR0B2), which is an atypical NR since it lacks a DNA-binding domain and acts as a corepressor inhibiting coactivator binding and directly repressing transcriptional activity of other NRs, such as liver receptor homolog-1 (LRH-1, NR5A2), hepatocyte nuclear factor 4 (HNF-4α), and retinoid X receptor (RXR) (Goodwin et al. 2000; Anakk et al. 2011; Kerr et al. 2002; Wang et al. 2002; Lee et al. 2000; Seol et al. 1996; Lee and Moore 2002; Lu et al. 2000). SHP is recruited to the promotor of target genes with the help of other NRs (Kliewer and Mangelsdorf 2015). HNF-4α and LRH-1 bind to the BA-response elements (BAREs) of the CYP7A1 and CYP8B1 promoters and recruit SHP to this site (Goodwin et al. 2000; Lu et al. 2000; Kliewer and Mangelsdorf 2015; Stroup et al. 1997; Yang et al. 2002; Zhang and Chiang 2001; Keitel et al. 2008). Targeted deletion of SHP impaired but did not completely abolish the BA-dependent suppression of CYP7A1, indicating that several redundant (and also SHP-independent) mechanisms facilitate BA-mediated repression of CYP7A1 transcription (Kerr et al. 2002; Wang et al. 2002). BA reuptake into enterocytes in the terminal ileum (see below) triggers activation of FXR and expression of its target gene fibroblast growth factor 15 (Fgf15, which corresponds to FGF19 in humans), which circulates to the liver via portal blood, where it binds to the FGF receptor 4 (FGFR4) on hepatocytes (Fig. 1) (Kliewer and Mangelsdorf 2015; Inagaki et al. 2005). Binding of Fgf15/FGF19 leads to dimerization of FGFR4 and autophosphorylation and activation of the c-Jun N-terminal kinase (JNK) pathway resulting in repression of CYP7A1 transcription (Holt et al. 2003). However, SHP is also required for efficient repression of CYP7A1 by Fgf15/FGF19, since overexpression of Fgf15 triggered only a minor, not significant reduction in Cyp7a1 mRNA levels in SHP KO mice (Kliewer and Mangelsdorf 2015; Inagaki et al. 2005). While mice with liver-specific targeted deletion of FXR retained the ability to suppress Cyp7a1 transcription when treated with the synthetic FXR agonist GW4064, mice with targeted deletion of FXR exclusively in the intestine were resistant to Cyp7a1 repression in response to GW4064 (Kim et al. 2007). In contrast suppression of Cyp8b1 was dependent on FXR in the liver, and recombinant Fgf15 application had no effect on Cyp8b1 mRNA levels while significantly lowering Cyp7a1 expression (Kliewer and Mangelsdorf 2015; Kim et al. 2007). Thus, activation of the FXR-Fgf15/FGF19 pathway in the intestine predominates over the FXR-SHP pathway in the liver in repression of CYP7A1 expression (Kliewer and Mangelsdorf 2015; Kim et al. 2007; Modica et al. 2012).

In hepatocytes bile is formed by ATP-dependent transport of bile constituents such as BAs, phospholipids, cholesterol, and bilirubin from the hepatocyte into the bile canaliculus. The bile salt export pump (BSEP, ABCB11) secretes BAs across the canalicular membrane, which is the major driving force for BA-dependent bile flow (Gerloff et al. 1998; Häussinger et al. 2004; Kullak-Ublick et al. 2004). Intracellular accumulation of BAs induces BSEP transcription via FXR enhancing their own secretion in a feed-forward manner. Organic anions and bilirubin conjugates are secreted into bile by MRP2 (ABCC2), while phospholipids are flopped from the inner to the outer leaflet of the canalicular membrane by MDR3
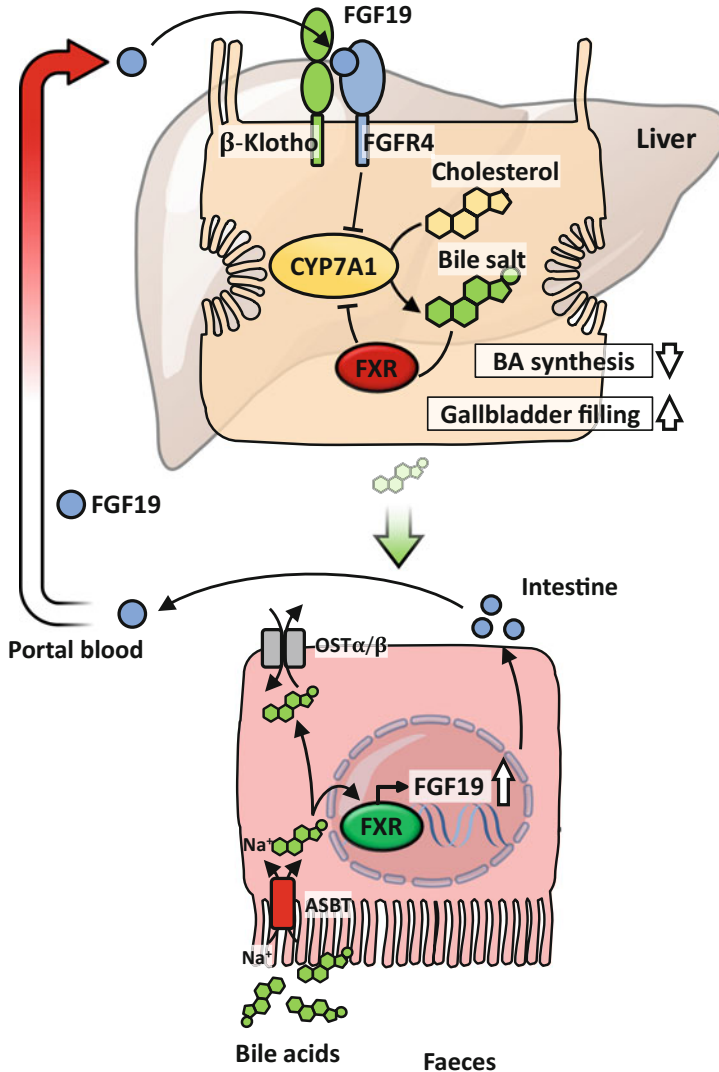
Fig. 1 FGF19 signaling in liver and intestine. Bile acids (BAs) are synthesized in the liver from cholesterol under control of CYP7A1. High hepatic BA concentrations activate FXR signaling thereby repressing CYP7A1 expression. In the intestine, FXR is activated by BAs resulting in increased FGF19 expression, which circulates to the liver and binds to FGF receptor 4 (FGFR4) and its coreceptor β-klotho on hepatocytes, resulting in suppression of CYP7A1 and reduced BA synthesis. Furthermore, FGF19 triggers gallbladder filling. For detailed description and references, refer to text

(ABCB4, Mdr2 in mice) (Smit et al. 1993; van Helvoort et al. 1996; Kamisako et al. 1999; Keppler et al. 2000). Both human multidrug resistance-associated protein

2 (MRP2, ABCC2) and the multidrug resistance protein 3 (MDR3, ABCB4) are at least in part positively regulated by FXR (Fig. 2) (Huang et al. 2003; Kast et al. 2002). Hence, FXR signaling triggers bile flow and secretion of cholephilic compounds (Keitel et al. 2008).

In cholangiocytes as well as in enterocytes, bile acid uptake is mediated by the apical sodium-dependent bile salt transporter (ASBT, SLC10A2) (Dawson et al. 2003; Lazaridis et al. 1997). ASBT transcription is differentially modulated between species with human ASBT expression being regulated by FXR (Fig. 2) (Kalaany and Mangelsdorf 2006; Neimark et al. 2004; Sinha et al. 2008). Basolateral export of BAs is facilitated in cholangiocytes as well as in enterocytes by the organic solute transporters α/β (OSTα/β, SLC51A/B) (Landrier et al. 2006; Wagner et al. 2003; Kazgan et al. 2014).

Hepatic BA uptake across the sinusoidal membrane is mediated via several transport proteins with more than 80% of conjugated BAs being imported by the sodium-dependent taurocholate cotransporting peptide (NTCP, SLC10A1) (Hagenbuch and Meier 1994; Kullak-Ublick 2003; Kullak-Ublick et al. 2000). Comparable to CYP7A1 and ASBT, BAs repress NTCP expression in a FXR-SHP-dependent mechanism (Denson et al. 2001; Jung et al. 2004). Nevertheless, further SHP-independent mechanisms of NTCP transcriptional regulation must exist, since NTCP mRNA levels in SHP KO mice were unaltered (Wang et al. 2002). Further BA uptake transporters in the basolateral hepatocyte membrane belong to the family of organic anion transporters (OATPs). While OATP1B1 expression is downregulated by FXR, OATP1B3 expression is increased (Fig. 2) (Jung and Kullak-Ublick 2003; Jung et al. 2002). The repression of BA uptake (via NTCP, OATP1B1 (Fig. 2)) in combination with suppression of BA de novo synthesis may protect hepatocytes from intracellular accumulation of toxic BAs under cholestatic conditions. Alternate BA secretion across the basolateral membrane into sinusoidal blood is facilitated by multidrug resistance-associated protein 4 (MRP4, ABCC4) and the organic solute transporter α/β (OSTα/β). Both transport systems are upregulated under cholestatic conditions in rodents and humans (Denk et al. 2004; Keitel et al. 2005; Schuetz et al. 2001; Boyer et al. 2006). While BAs induce the expression of OSTα/β via FXR (Fig. 2), the upregulation of MRP4 by BAs is FXR-independent and is observed both on the translational and posttranslational levels (Wagner et al. 2003; Schuetz et al. 2001; Boyer et al. 2006).

In hepatocytes, BAs activate FXR which subsequently inhibits de novo BA synthesis, enhances conjugation and detoxification, and increases BA efflux both across the canalicular and basolateral membranes. In the terminal ileum, FXR triggers expression of Fgf15/FGF19 which via FGFR4 on hepatocytes suppresses BA synthesis (Fig. 1). Therefore, FXR sensing and signaling in both, the liver and intestine, prevents BA overload and thus liver damage (Kliewer and Mangelsdorf 2015; Keitel et al. 2008).
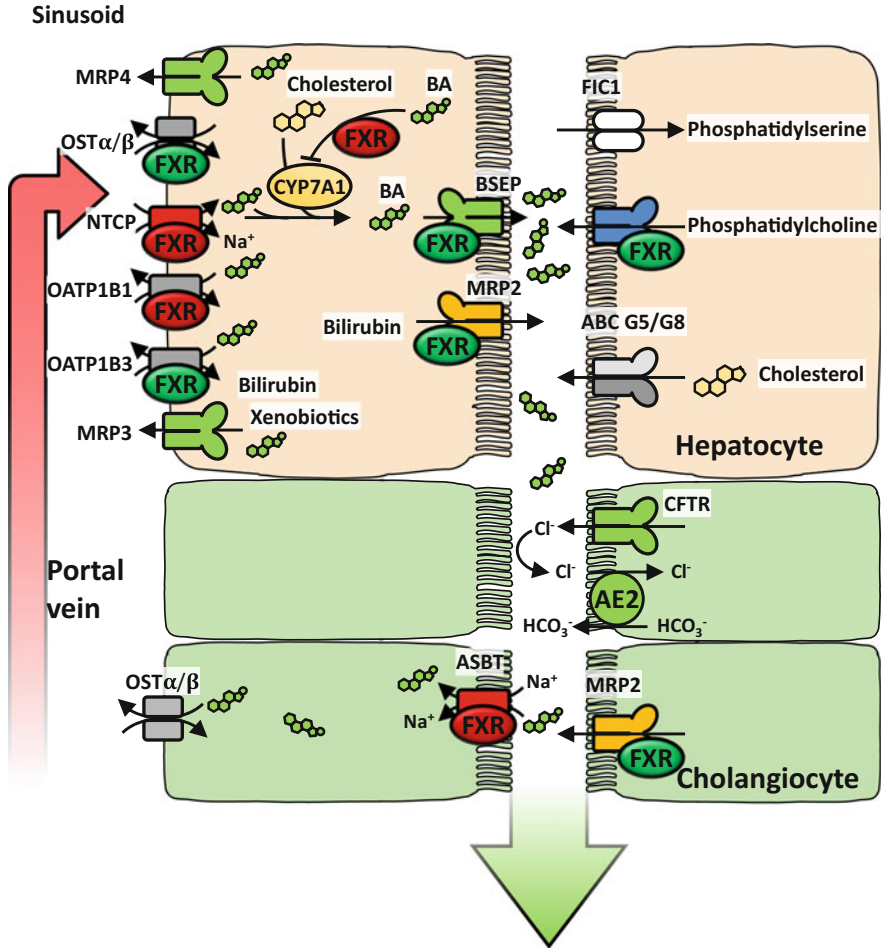
Fig. 2 Bile formation and regulation by FXR. Bile acids (BAs) are synthesized from cholesterol within hepatocytes and secreted by the bile salt export pump (BSEP) across the apical/canalicular membrane into the bile canaliculus. BAs form mixed micelles together with phosphatidylcholine transported by the phospholipid floppase MDR3 and cholesterol exported by ABCG5/G8. The ATPase FIC1 is a phosphatidylserine flippase maintaining membrane asymmetry. CFTR exports chloride from cholangiocytes which is subsequently exchanged against bicarbonate via the anion exchanger 2 (AE2), leading to formation of the bicarbonate umbrella, which protects cholangiocytes from toxic BA concentrations. ASBT acts as symporter for BAs and sodium, facilitating uptake of BAs into cholangiocytes. The organic solute transporter OSTα/β secretes BAs across the basolateral membrane of both cholangiocytes and hepatocytes. Different transport proteins are located in the basolateral/sinusoidal membrane of hepatocytes. The main importer for BAs is the sodium taurocholate cotransporting peptide (NTCP). However, both OATP1B1 and OATP1B3 can facilitate uptake of BAs and organic anions into hepatocytes. MRP4 and OSTα/β act as alternate BA exporters under cholestatic conditions. Bilirubin is mainly imported via OATP1B3 and transported across the canalicular membrane into bile by MRP2. Under cholestatic conditions bilirubin can be exported into sinusoidal blood via MRP3. FXR represses (red) de novo BA synthesis, hepatic BA uptake, and uptake of BAs into cholangiocytes via ASBT. In contrast, export mechanisms are positively regulated (green) by FXR. For a detailed description and references, refer to text

2   FXR Knockout Mice Are More Susceptible to Cholestasis

Mice with targeted deletion of Fxr displayed no obvious phenotype when held under standard housing conditions and fed a regular chow diet (Sinal et al. 2000). However, lack of Fxr resulted in increased (about eightfold) levels of serum BAs. Supplementation of the diet with 1% cholic acid (CA) led to wasting, hypothermia, and an increased mortality of about 30% by day 7 in the Fxr knockout mice (KO), none of which was observed in the wild-type animals (Sinal et al. 2000). Serum BA levels were 23-fold higher in Fxr KO mice than in wild-type animals, which may be attributed to the impaired secretion of BAs into bile resulting in accumulation of BAs within hepatocytes, increased elimination of BAs across the basolateral membrane, and thus reduced excretion into feces (Sinal et al. 2000). Hepatic BA overload is aggravated in Fxr KO mice by impaired suppression of Cyp7a1 and Cyp8b1 mRNA levels and failure to upregulate Bsep expression in response to CA feeding (Sinal et al. 2000; Zollner et al. 2003). However, the overall BA pool was reduced in the absence of FXR both in chow-fed and in CA-fed cohorts. Upregulation of the detoxification enzyme Cyp3a11 increased BA hydroxylation and enhanced secretion into urine via Mrp4, which represents a protective adaptive mechanism in Fxr KO mice (Cho et al. 2010; Marschall et al. 2006). In contrast to CA feeding, common bile duct ligation (CBDL), which serves as model of extrahepatic obstructive cholestasis, was associated with similar mortality rates in Fxr KO and wild-type animals (Wagner et al. 2003). Overall, Fxr KO mice were relatively resistant toward CBDL-induced liver damage (Wagner et al. 2003; Stedman et al. 2006). Potential mechanisms of protection in this experimental setting are the lower BA concentrations in Fxr KO mice as well as the reduced expression of Bsep, which result in diminished bile flow, reduced biliary pressure, less bile infarcts, and thus reduced liver injury (Wagner et al. 2003; Stedman et al. 2006). Histologically livers from Fxr KO mice showed disseminated necrosis as compared to wild-type livers, which displayed marked bile infarcts after CBDL (Wagner et al. 2003; Stedman et al. 2006). Interestingly, upregulation of Mrp2, Mrp3, and Mrp4 as a consequence of CA feeding or CBDL was independent of Fxr in mice livers, while induction of Bsep expression was strictly dependent on Fxr (Wagner et al. 2003; Zollner et al. 2003).

Alpha-naphthylisothiocyanate (ANIT) administration recapitulates a cholangio-cellular intrahepatic cholestasis. Hepatocytes facilitate GSH conjugation of ANIT, which can then be secreted into bile via Mrp2 (Abcc2), where GSH dissociates from ANIT leading to biliary injury (Cui et al. 2009; Dietrich et al. 2001). Similar to BA overload by feeding and CBDL, ANIT-treated Fxr KO mice failed to induce Bsep and Ostα expression and displayed impaired suppression of Ntcp mRNA (Cui et al. 2009). Conversely, intraperitoneal injection of the FXR agonist GW4064, prior to oral ANIT administration, ameliorated liver injury in wild-type mice (Cui et al. 2009). These findings underscore the essential role of Fxr signaling in protection from cholestatic liver injury.

Fxr and Shp double-knockout mice (DKO) were generated and were expected to phenocopy Fxr KO mice under the assumption that SHP is an exclusive downstream target of FXR (Anakk et al. 2011). In contrast to Fxr KO mice, Fxr-Shp DKO mice

displayed hepatomegaly, increased hepatocyte proliferation, hepatocyte necrosis, and ductular proliferation resulting in elevated serum levels of AST, ALT, bilirubin, and BAs. These changes became evident as early as 3 weeks of age (Anakk et al. 2011). A strong induction of both Cyp7a1 and Cyp8b1 was observed, while Cyp27a1 was significantly reduced, consistent with increased BA synthesis resulting in elevation of hepatic and serum BA levels (Anakk et al. 2011). Furthermore, Bsep was downregulated in livers of DKO mice contributing to retention of BAs within the hepatocyte and thus hepatocyte injury (Anakk et al. 2011). Expression of basolateral BA efflux transporters Mrp3 and Mrp4 was induced, while mRNA levels of basolateral BA uptake transporters Ntcp and Oatp1 were reduced in DKO mice as an adaptive mechanism to counteract BA overload within the hepatocyte. In contrast to single-knockout mice for Fxr, Shp, or Bsep, these DKO phenocopy multiple features of children with progressive familial intrahepatic cholestasis (PFIC) and thus serve as a model for early-onset intrahepatic cholestasis syndromes (Sinal et al. 2000; Anakk et al. 2011; Wang et al. 2001, 2002, 2003).

3 Genetic Variants in Human FXR Are Associated with a Spectrum of Cholestatic Disorders

Genetic variants in the FXR gene were first identified in women with intrahepatic cholestasis of pregnancy (ICP) (van Mil et al. 2007). ICP is the most common liver disease in pregnant women and affects 0.1–2.0% of pregnancies in Europe (Bacq 2011; Joshi et al. 2010; Lammert et al. 2000; Wikstrom Shemer et al. 2013; Keitel et al. 2016; Williamson and Geenes 2014). ICP usually presents with pruritus, elevated serum BAs, and transaminase levels late in pregnancy (up to 80% become clinically evident after 28 weeks of gestation) (Lammert et al. 2000; Keitel et al. 2016; Williamson and Geenes 2014; Bacq et al. 2012). Perinatal complications such as spontaneous or iatrogenic premature delivery, meconium staining of the amniotic fluid, respiratory distress, low Apgar scores, and even stillbirth are more frequent in women with ICP, especially if maternal BA levels exceed 40 μmol/l (Keitel et al. 2016; Geenes et al. 2014; Glantz et al. 2004; Rioseco et al. 1994; Williamson et al. 2004). Analysis of long-term outcome of women, who had previous ICP, demonstrated an increased incidence of various liver, biliary, pancreatic, metabolic, and immune-mediated diseases, including nonalcoholic liver cirrhosis, cholelithiasis, cholecystitis and cholangitis, gallstone-associated pancreatitis, hepatocellular and biliary cancer, as well as diabetes and cardiovascular and thyroid disease (Keitel et al. 2016; Marschall et al. 2013; Ropponen et al. 2006; Wikstrom Shemer et al. 2015). ICP is precipitated in genetically susceptible women by gestational hormones as well as environmental factors, which are not fully understood (Lammert et al. 2000). While most of the genetic risk for ICP has been linked to variants in the MDR3 (ABCB4) gene, also variants associated with ICP have been identified in the genes encoding BSEP (ABCB11), FIC1 (ATP8B1),

MRP2 (ABCC2), tight junction protein 2 (TJP2), as well as FXR (NR1H4) (van Mil et al. 2007; de Vree et al. 1998; Dixon et al. 2009, 2014, 2017; Gudbjartsson et al. 2015; Jacquemin et al. 1999; Müllenbach et al. 2003, 2005; Pauli-Magnus et al. 2004; Wasmuth et al. 2007; Keitel et al. 2006). Overall a study of FXR genetic contribution to ICP identified four variants: c.-1G>T (rs56163822, MAF 4.45%), c.1A>G (p.M1V, rs138943609, MAF 0.023%), c.238T>C (p.W80R), and c.518T>C (p.M173T, rs6155050, MAF 0.39%), of which c.-1G>T and c.518C>T were also present in the respective control cohort (van Mil et al. 2007). (Reference sequence for FXR is NM_001206979.1; minor allele frequencies (MAF) were taken from http://gnomad.broadinstitute.org/.) The variants c.-1G>T, c.1A>G (p.M1V), and c.518C>T (p.M173T) resulted in reduced target gene expression in vitro (van Mil et al. 2007). In contrast to BSEP (ABCB11) and MDR3 (ABCB4), variants in FXR are less frequently detected in ICP.

Recently four individuals from two families were identified, who carried either a homozygous truncation variant (c.526C>T, p.R176*) within the FXR DNA-binding domain or a homozygous in-frame insertion variant c.419_420insAAA (p.Y139_N140insK), resulting in a 31.7 kb deletion affecting the zinc-binding module of the FXR DNA-binding domain (Gomez-Ospina et al. 2016). Immunohistochemistry of the patients' livers revealed complete absence of FXR and BSEP staining, while MDR3 could be detected in all livers (Gomez-Ospina et al. 2016). This finding again underscores the strict dependency of BSEP expression on the presence of FXR as suggested by rodent studies (Wagner et al. 2003). All four patients developed clinically apparent signs of jaundice, cholestasis, and liver damage within the first 6 weeks of life, while the parents carrying only one affected allele were asymptomatic (Gomez-Ospina et al. 2016). Liver disease was rapidly progressive in the affected children resulting in early death of both children with the in-frame insertion at age of 5 weeks and 8 months, respectively, and the need for liver transplantation at the age of 4.4 months and 22 months of the patients with the truncation variant (Gomez-Ospina et al. 2016). The absence of BSEP in these children partially phenocopies the phenotype of BSEP deficiency (PFIC2) with normal to low serum levels for gamma-glutamyltransferase (GGT) despite elevated AST, ALT, and bilirubin levels. However, FXR deficiency was characterized by vitamin K-independent coagulopathy, high AFP serum levels, and reduced FGF19 levels (Gomez-Ospina et al. 2016). The latter are explained by absence of FXR from the intestine. After successful liver transplantation, the two affected children now express wild-type FXR only in the donor liver and not in other organs such as the intestine, kidney, or adrenal glands (Gomez-Ospina et al. 2016). During the observed time period after transplantation (about 8 years in patient 1 and 11 months in patient 2), no overt pathology became apparent in other organs (Gomez-Ospina et al. 2016). Therefore, it will be interesting to monitor these patients for signs of extrahepatic FXR deficiency in the future.

4 FXR Expression and Function Is Altered in Different Forms of Intrahepatic Cholestasis

Altered FXR expression has been observed not only in patients with severe genetic variants in the FXR gene but also in patients with PFIC1 disease due to variants in the FIC1 (ATP8B1) gene. Absence of FIC1, which is a hallmark of severe PFIC1, was associated with reduced FXR activity and FXR expression resulting in impaired target gene transactivation in an intestinal cell line (Chen et al. 2004). While the experiments of this study were restricted to intestinal changes, reduced BSEP expression in the liver may contribute to the phenotype and explain the similar clinical presentation of FIC1- and BSEP-related PFIC subtypes (PFIC1 and PFIC2, respectively) (Chen et al. 2004). Whether the downregulation of FXR in PFIC1 is a direct or an indirect effect of cholestasis remains elusive (Cai et al. 2009).

As described above ICP is associated with altered BA homeostasis and elevated maternal serum BA levels (Milona et al. 2010). Elevation of 17β-estradiol and its metabolites during pregnancy impairs transactivation of FXR target genes, such as Cyp7a1, Cyp8b1, or Bsep, through estrogen receptor α (ERα) (Milona et al. 2010). ERα in turn directly interacts with FXR on the protein level, thus preventing the binding of FXR to the FXR-response element in the target gene promotor (Milona et al. 2010; Song et al. 2014). In addition, sulfated progesterone metabolites, which are elevated in normal pregnancy and further raised in ICP patients, also impair FXR activation and target gene expression (Keitel et al. 2016; Abu-Hayyeh et al. 2013, 2016; Abu-Hayyeh and Williamson 2015). Attenuated FXR signaling may therefore contribute to hypercholanemia, dyslipidemia, and gallstone formation during pregnancy (Keitel et al. 2016; Abu-Hayyeh et al. 2013; Abu-Hayyeh and Williamson 2015).

5 Targeting FXR in Cholestasis: Lessons from Rodents

Disruption of Fxr or combined deletion of Fxr and Shp triggers development of cholestasis of varying severity, while stimulation of Fxr promotes transcription of BA detoxification enzymes and hepatobiliary transport proteins facilitating BA clearance and represses enzymes relevant for BA synthesis thus lowering BA levels in hepatocytes (Sinal et al. 2000; Anakk et al. 2011; Marschall et al. 2006; Guo et al. 2003; Liu et al. 2003; Ananthanarayanan et al. 2001). Therefore, activation of FXR signaling with BA and non-BA ligands has emerged as attractive therapeutic target for different cholestatic liver diseases.

Intraperitoneal injection of 17α-ethinylestradiol ($E_2$17α) for 5 days mimics intrahepatic cholestasis induced by drugs or pregnancy (ICP). Bile flow was lowered by about 50% after 5 days of $E_2$17α injection (Fiorucci et al. 2005). Simultaneous administration of the CDCA-derived FXR agonist 6-ethyl chenodeoxycholic acid (6-ECDCA, also known as INT7-747 or obeticholic acid (OCA)) or the synthetic agonist GW4064 completely restored the $E_2$17α-induced reduction in bile flow (Fiorucci et al. 2005). The increase in bile flow in response to OCA or GW4064 was accompanied by an upregulation of Bsep, Mrp2, and Mdr2 mRNA expression in

liver tissue of these rats (Fiorucci et al. 2005). In a further model, intraperitoneal administration of GW4064 protected against alpha-naphthylisothiocyanate induced cholangiocellular cholestasis in both mice and rats (Cui et al. 2009; Liu et al. 2003). In rats, intraperitoneal GW4064 injection about 24 h prior to a single oral dose of ANIT significantly reduced serum levels of AST, ALT, LDH, alkaline phosphatase (ALP), and BAs, which was accompanied by a significant induction of Bsep, Mdr2, Mrp2, and SHP expression in liver tissue (Liu et al. 2003). Injection of GW4064 to rats resulted in a significant upregulation of Mdr2 (Abcb4), Bsep (Abcb11), and SHP mRNA expression in liver tissue of these animals (Liu et al. 2003). Upregulation of BSEP, MDR3, and SHP was also observed in human hepatocytes after 12 h of GW4064 stimulation (Liu et al. 2003). Injection of GW4064 24 h after ligation of the common bile duct (CBDL) significantly reduced serum levels for AST, ALT, and LDH but not for ALP, BAs, or bilirubin as compared to vehicle-treated CBDL rats. Liver histology revealed lower numbers of bile infarcts in GW4064-treated CBDL rats as compared to CBDL alone (Liu et al. 2003). However, the beneficial effect of Fxr activation in obstructive cholestasis has been controversial, since Fxr KO mice are relatively protected from liver damage induced by CBDL (Wagner et al. 2003; Stedman et al. 2006). This conflict that both targeted deletion and systemic stimulation of Fxr protect from obstructive cholestasis may relate to the effects of Fxr signaling in the liver and intestine. Liver damage in response to CBDL results from increased pressure in the biliary tree and retention of bile acids within the liver (Wagner et al. 2003). Inhibition of BA synthesis and stimulation of basolateral efflux of bile constituents from the hepatocyte represent protective mechanisms against BA overload (Wagner et al. 2003; Stedman et al. 2006). These mechanisms are already induced in Fxr KO mice but can also be triggered by transactivation of Fxr in the intestine leading to Fgf15-mediated endocrine suppression of hepatic BA synthesis (Modica et al. 2012; Wagner et al. 2003; Keitel et al. 2005; Stedman et al. 2006). Overexpression of Fxr in the intestine resulted in upregulation of Fxr target gene expression such as Fgf15, Shp, Ostα, and Ostβ in the terminal ileum (Modica et al. 2012). In the liver a complete repression of Cyp7a1 and an about 30% reduction in BA pool size were observed in these animals (Modica et al. 2012). CBDL in mice overexpressing Fxr in the intestine ameliorated liver damage as measured by reduced levels for AST, ALT, ALP, BAs, and bilirubin as well as less bile infarcts (Modica et al. 2012). Besides the pronounced protective effect on the liver, Fxr overexpression inhibited bacterial overgrowth and translocation and promoted intestinal barrier integrity (Modica et al. 2012; Inagaki et al. 2006). Overexpression of Fxr in the intestine also protected from ANIT-induced cholestasis (Modica et al. 2012). Mdr2 (Abcb4) KO mice are characterized by defective phospholipid secretion into bile exposing cholangiocytes to toxic levels of BAs, which results in biliary damage and progressive sclerosing cholangitis (Fickert et al. 2002, 2004). Mdr2 KO mice are commonly used as model for primary sclerosing cholangitis (PSC) and ABCB4 (MDR3)-related disease (Fickert et al. 2002, 2004). Overexpression of Fxr in the intestine of Mdr2 KO mice reversed and attenuated liver damage in these animals as shown by reduced serum levels of AST, ALT, ALP, and BAs as well as on histology (Modica et al. 2012). In contrast double-knockout mice for Mdr2 and Fxr suffered

from aggravated liver damage with severe elevation of AST, ALT, ALP, and BAs (Modica et al. 2012). This finding is in line with the report that feeding of INT-767 (6α-ethyl-3α,7α,23-trihydroxy-24-nor-5 β-cholan-23-sulfate), which is a dual agonist for FXR as well as for the G protein-coupled bile acid receptor TGR5 (GPBAR1), reduced liver injury in Mdr2 KO mice; however, the beneficial effects were almost exclusively attributed to Fxr activation (Baghdasaryan et al. 2011).

Intraperitoneal injection of recombinant human FGF19 efficiently suppressed Cyp7a expression and protein levels and lowered the BA pool by 30% in wild-type mice (Modica et al. 2012). Administration of recombinant FGF19 to wild-type mice 4 days prior to CBDL ameliorated liver injury, demonstrating that FGF19 protects against cholestasis through the reduction in BA synthesis and BA pool size (Modica et al. 2012). Thus, the FXR target gene Fgf15/FGF19 alone seems to be sufficient for treatment of different cholestatic disorders (Modica et al. 2012).

FGF19 and its receptor fibroblast growth factor receptor 4 (FGFR4) are not only relevant for normal liver regeneration but have been linked to hepatocarcinogenesis in both rodents and humans (Alvarez-Sola et al. 2017; Uriarte et al. 2013, 2015; Padrissa-Altes et al. 2015; Zhou et al. 2014). Transgenic overexpression of human FGF19 in skeletal muscle resulted in HCC development by the age of 10 months in 80% of female mice (Nicholes et al. 2002). Using an adeno-associated virus (AAV)-mediated gene delivery system via tail vein injection, overexpression of human FGF19 could be achieved in the liver and induced HCC development in mice in a strain-dependent manner in up to 100% of animals (Zhou et al. 2014). Induction of hepatocarcinogenesis in Fgf15 wild-type and KO mice using diethylnitrosamine (DEN) and carbon tetrachloride (CCL4) injection led to more pronounced fibrosis and tumor development in Fgf15 wild-type mice as compared to KO mice (Uriarte et al. 2015). In humans, focal amplifications of the FGF19 gene have been observed in about 15% of HCCs, overexpression of FGF19 was found in about 25% of HCCs, while high levels of FGFR4 expression were present in 30–50% of HCCs, further underscoring the relevance of FGF19-FGFR4 signaling in hepatocarcinogenesis (Alvarez-Sola et al. 2017; Ho et al. 2009; Sawey et al. 2011).

Conversely, long-term administration of dual FXR/TGR5 agonist INT-767 to Mdr2 KO mice improved liver injury and prevented spontaneous HCC development (Cariello et al. 2017). This beneficial effect was not observed in Fxr KO mice, which are also prone to spontaneous HCC development underscoring that FXR signaling is essential for the protection of Mdr2 KO mice (Cariello et al. 2017). The mechanisms why activation of FXR pathways can both prevent and promote tumor development remain unclear at the moment.

Since FGF19-FGFR4 signaling has been implicated in HCC tumorigeneses, safety concerns have been raised regarding FXR- and FGF19-based therapies especially in precancerous conditions such as advanced liver fibrosis, nonalcoholic steatohepatitis, or primary sclerosing cholangitis (PSC). Deletion of 5 amino acids (P24-S28) and introduction of 3 amino acid substitutions (A30S, G31S, and H33L) resulted in a FGF19 variant (denoted as M70, later named NGM282), which enables CYP7A1 suppression but prevents the proliferative and tumorigenic signaling. M70 cannot trigger phosphorylation and activation of signal transducer and activator of

transcription 3 (STAT3) and its target genes cyclin D1 and survivin (Zhou et al. 2014). In contrast, suppression of CYP7A1 and reduction in serum BA levels were comparable to wild-type FGF19 (Zhou et al. 2014). Therefore, this FGF19 variant (M70) was tested in animal models of cholestasis. Twice daily subcutaneous injection of M70 starting 4 days prior to CBDL prevented liver injury and lowered serum AST, ALT, ALP, bilirubin, and BA levels (Luo et al. 2014). On liver histology M70 treatment was associated with fewer and smaller bile infarcts when compared to vehicle-treated CBDL mice (Luo et al. 2014). A similar beneficial effect of M70 was observed in ANIT-induced cholangiocellular cholestasis (Luo et al. 2014). M70 was administered twice daily subcutaneously for 4 days prior to oral ANIT application (Luo et al. 2014). With regard to changes in gene expression, M70 suppressed Cyp7a1 and Bsep mRNA levels to similar extent as wild-type FGF19 (Luo et al. 2014). Overall, the beneficial effect of M70 was indistinguishable from wild-type FGF19 in these models of cholestasis (Luo et al. 2014).

Treatment of 12-week-old Mdr2 KO mice with FGF19 or M70 by AAV injection not only inhibited expression of Cyp7a1, Cyp27a1, and Bsep and thus cholestasis but also significantly reduced hepatic inflammation, biliary fibrosis, and overall liver injury (Zhou et al. 2016). Furthermore, while FGF19 treatment led to the formation of HCCs by week 32, no HCCs were observed in livers of M70-treated Mdr2 KO mice. M70 also reduced ductular proliferation and ameliorated hepatosplenomegaly in Mdr2 KO mice with established cholangiopathy, thus reverting the phenotype of these mice (Zhou et al. 2016). This finding suggested a potential application of M70 in MDR3 disease (PFIC3) as well as for cholangiopathies.

Administration of M70 to healthy volunteers subcutaneously for 7 days resulted in a significant reduction of serum C4 levels as marker for CYP7A1 activity, demonstrating feasibility of targeting FGF19 signaling in humans (Luo et al. 2014).

6 Targeting FXR Signaling in Cholestasis: Current and Future Clinical Applications

Since agonists of FXR as well as its downstream target FGF19 were beneficial in different rodent models of intra- and extrahepatic cholestasis, different FXR modulators as well as a nontumorigenic FGF19 analogue (M70, NGM282) have been developed. The first clinical trials with FXR ligands and the FGF19 analogue (M70, NGM282) were carried out in patients with primary biliary cholangitis (PBC) and primary sclerosing cholangitis (PSC).

6.1 Treatment of Primary Biliary Cholangitis (PBC) with FXR Agonists and an FGF19 Analogue

PBC affects predominantly middle-aged women, has an incidence of about 5 in 100,000 inhabitants per year, and is characterized histologically by a chronic, nonsuppurative inflammation of the small intrahepatic bile ducts, which can result

in the destruction of the bile ducts, in the development of portal fibrosis and ultimately cirrhosis and its complications (Boonstra et al. 2012a; Carey et al. 2015). Transplant-free survival of untreated PBC ranges around 6–10 years (Carey et al. 2015). Ursodeoxycholic acid (UDCA) in a dose of 13–15 mg/kg BW is recommended as first-line treatment for all PBC patients since it has been shown to lower serum liver tests but also to improve liver histology, it delays disease progression, and it improves transplant-free survival (Beuers et al. 2015; Corpechot et al. 2008, 2011; European Association for the Study of the Liver 2017; Hirschfield et al. 2018a; Leuschner et al. 1996; Poupon et al. 1991, 1999). However, depending on which combination of laboratory values and thus which scoring system is used, only 26–86% of PBC patients show a positive response to UDCA at 12 months after starting treatment (Corpechot et al. 2008, 2011; Lammers et al. 2015; Kuiper et al. 2009; Pares et al. 2006). Thus, 30–50% of PBC patients have an inadequate UDCA response and are at risk of disease progression. In addition, there is a small group of patients who cannot tolerate UDCA treatment. These two groups of patients have been in need for further therapies (Bahar et al. 2018).

The FXR agonist obeticholic acid (OCA, formerly 6-ECDCA, INT-747) was evaluated in phase II studies for PBC, both as monotherapy and in combination with UDCA (Kowdley et al. 2018; Hirschfield et al. 2015). Patient selection comprised treatment-naive patients and those with insufficient laboratory response to UDCA. Treatment was carried out over 12 weeks with different OCA doses (10 mg up to 50 mg/day) (Kowdley et al. 2018; Hirschfield et al. 2015). In both trials, OCA triggered a reduction in ALP values from the baseline, which was significant as compared to the placebo group (Kowdley et al. 2018; Hirschfield et al. 2015). In the phase III study (POISE), 216 PBC patients with intolerance toward UDCA and/or inadequate response to UDCA and ALP levels above 1.67 times the upper limit of normal (ULN) and/or bilirubin levels (between 1 and 1.9-times ULN) were randomized to placebo, OCA at 10 mg daily, or OCA at 5 mg daily. The 5 mg OCA group could be titrated to 10 mg after 24 weeks depending on the response (Nevens et al. 2016). Over 90% of the study participants received UDCA maintenance therapy (Nevens et al. 2016). The primary endpoint of the study was a drop in ALP values below 1.67 times the ULN after 12 months of therapy. This goal was achieved by 46% in the 5–10 mg OCA group, by 47% in the 10 mg OCA group, and also by 10% of the placebo-treated patients (Nevens et al. 2016). In 77% of patients in the two OCA treatment groups, ALP values fell by more than 15%, which was the secondary endpoint of the study (Nevens et al. 2016). This endpoint was also reached by 29% of the patients in the placebo group. The most common side effect of OCA was itching, which was dose-related and was the cause of premature discontinuation of the study by eight patients corresponding to about 10% of the OCA-treated patients (Nevens et al. 2016). This OCA-specific adverse effect was already known from phase II studies, which is why a lower OCA starting dose was used in the phase III trial as compared to the phase II trials (Kowdley et al. 2018; Hirschfield et al. 2015; Nevens et al. 2016). Based on this data, FDA and EMEA

granted conditional approval of OCA in PBC patients with inadequate response or intolerance to UDCA. The recommended starting dose of OCA is 5 mg daily, which should be titrated up to 10 mg daily after 3 months depending on decrease in ALP values and side effects, especially itch intensity.

Despite the fact that the clinical trials did not include patients with decompensated liver cirrhosis (Child-Pugh stages B and C), a dosing recommendation for these patients was included, starting with 5 mg once weekly and titration up to 10 mg twice weekly (Bahar et al. 2018). After marketing approval, safety announcements have been issued relating to 19 PBC patients with Child-Pugh B/C cirrhosis, who had received daily instead of weekly dosing and developed subsequent liver injury or even death (Bahar et al. 2018). Based on data from the phase III study, it can be assumed that about 50% of patients with inadequate response to UDCA will decrease ALP levels below 1.5 to 1.67 times the ULN and thus experience a significant improvement of prognosis; however, long-term trials and real-world data will have to confirm whether these beneficial effects can be translated into clinical routine (Bahar et al. 2018). However, even with addition of OCA, some PBC patients will not lower ALP values sufficiently and thus require further treatment options. The main unwanted effect of OCA is pruritus, which is dose-dependent and may be partially explained by activation of the membrane-bound G protein-coupled bile acid receptor TGR5. Taurine- and glycine-conjugated OCA have an EC_{50} of 0.2 and 0.3 μM for TGR5 activation, respectively, which should be reached in patients, since OCA undergoes enterohepatic circulation and is enriched in the BA pool (Tully et al. 2017).

Further non-bile acid FXR modulators, including cilofexor (formerly GS-9674, Px-104) and tropifexor (formerly LJN452), are currently evaluated in phase II trials in PBC patients (Bahar et al. 2018; Massafra et al. 2018).

The FGF19 analogue NGM282 (formerly M70) has been investigated in a randomized, double-blind, placebo-controlled phase II trial in PBC patients with inadequate response to UDCA over 28 days (Mayo et al. 2018). Overall, 45 patients were randomized to receive subcutaneous daily doses of 0.3 mg or 3 mg NGM282 or placebo (Mayo et al. 2018). Almost 50% of patients receiving NGM282 achieved a 15% or greater reduction in serum ALP levels as compared to baseline, while only 7% of patients in the placebo group reached this endpoint by day 28 (Mayo et al. 2018). Moreover, AST and ALT levels were significantly reduced by NGM282 treatment, as were C4 levels for the 3 mg treatment group. Discontinuation of study medication resulted in an increase in ALP, AST, ALT, and GGT values, which returned to baseline values (Mayo et al. 2018). NGM282 was well tolerated with most adverse events being classified as grade I and grade II and similar to placebo-treated patients. However, diarrhea and loose stools were more frequent in the NGM282 treatment groups (Mayo et al. 2018). Changes in pruritus severity or quality of life as assessed by the PBC-40 questionnaire during the study period were comparable between patients receiving either NGM282 or placebo (Mayo et al. 2018).

6.2   Treatment of Primary Sclerosing Cholangitis (PSC) with FXR Agonists and an FGF19 Analogue

PSC affects predominantly men with a median age at diagnosis around 40 years, has an incidence of about 1 in 100,000 persons per year, and is characterized histologically by a chronic inflammation and fibro-obliterative destruction of intra- and/or extrahepatic bile ducts, resulting in progressive biliary fibrosis, cirrhosis, and its complications (Boonstra et al. 2012b; Lazaridis and LaRusso 2016). PSC is strongly associated with inflammatory bowel disease (up to 80% of patients have both conditions) and is a predisposing factor for colon, cholangiocellular, and gallbladder cancer (Lazaridis and LaRusso 2016; Eaton et al. 2013; Hirschfield et al. 2013). The FXR agonist OCA has recently been evaluated in a phase II placebo-controlled double-blind trial in patients with PSC. The data obtained in this trial is not published yet; however, preliminary results have been added to clinicaltrials.gov (NCT02177136). Overall, 77 PSC patients were randomized to receive either placebo, 1.5 mg OCA daily titrated to 3 mg after 12 weeks, or 5 mg OCA titrated to 10 mg after 12 weeks. The overall trial duration was 24 weeks. In both OCA treatment groups, a significant reduction in ALP values was observed as compared to baseline and placebo.

Cilofexor (formerly GS-9675, Px104) is an orally available selective (EC_{50} 43 nM) nonsteroidal agonist for FXR, which was evaluated in a randomized, placebo-controlled phase II trial over 12 weeks in PSC (Trauner et al. 2019). Fifty-two patients with large-duct PSC as demonstrated by magnetic resonance cholangiopancreatography or endoscopic retrograde cholangiopancreatography within the previous 12 months and ALP levels greater than 1.67 times the ULN were randomized to receive placebo, cilofexor 30 mg once daily, or cilofexor 100 mg once daily for 12 weeks. Randomization was stratified to the presence of UDCA maintenance therapy. The study offered a 96-week open-label extension with 100 mg cilofexor daily to all patients completing the 12 weeks of blinded treatment (Trauner et al. 2019). Treatment with cilofexor showed a significant (for the 100 mg dose) and dose-dependent decrease in ALP values as compared to baseline (−21% relative ALP reduction for 100 mg, −6% relative ALP reduction for 30 mg), which was not observed in placebo-treated patients (+3% ALP relative increase versus baseline) (Trauner et al. 2019). Treatment with cilofexor also resulted in a dose-dependent significant reduction of AST, ALT, and GGT values from baseline. Overall, cilofexor was well tolerated, and only three patients (14%) discontinued drug treatment. A significant reduction in serum BA levels as well as of C4 levels was observed in the 100 mg cilofexor group (Trauner et al. 2019). Pruritus was observed in 14–20% of patients treated with cilofexor, while 40% of patients in the placebo group reported pruritus (Trauner et al. 2019). Importantly, bowel disease remained stable during the 12 weeks trial (Trauner et al. 2019). Larger studies with FXR agonists are needed to evaluate whether the reduction in ALP will result in a clinically meaningful outcome improvement for PSC patients and to determine potential long-term side effects, especially with regard to malignancy development.

The FGF19 analogue (NGM282, formerly M70) has also been recently evaluated in a randomized, double-blind, placebo-controlled phase II trial in PSC patients (Hirschfield et al. 2018b). Sixty-two patients were randomized to receive placebo, NGM282 1 mg or 3 mg per subcutaneous injection once daily for 12 weeks. The primary outcome was the change in ALP levels from baseline at 12 weeks. Neither dose of NGM282 led to significant reduction in ALP levels from baseline by week 12 (Hirschfield et al. 2018b). However, NGM282 treatment resulted in a dose-dependent highly significant reduction of BA synthesis (C4 levels) as well as serum BA levels. Furthermore, AST and ALT values were significantly lower at end of treatment in the 3 mg NGM282 group (Hirschfield et al. 2018b). Furthermore, NGM282 significantly and dose-dependently improved serum markers for liver fibrosis (ELF score and Pro-C3) as compared to placebo-treated patients (Hirschfield et al. 2018b). 81–95% of patients treated with NGM282 experienced adverse effects, most of which were grade 1 or grade 2 and were mainly related to injection site or diarrhea. Three patients reported serious adverse events, one of which was potentially related to NGM282 (bowel obstruction in the follow-up period, which resolved) (Hirschfield et al. 2018b). Overall, the drug was well tolerated. Antibodies against FGF19 were not found in any of the patients, who tested positive for antidrug antibodies (Hirschfield et al. 2018b). Further trials in PSC will be needed to determine if ALP reduction is an adequate study endpoint. Also, longer trials will be needed to determine if the anti-fibrotic effects as well as the changes in BA metabolism will prevent disease progression and improve outcome in PSC patients treated with NGM282 (Hirschfield et al. 2018b).

7 Summary and Perspectives

FXR and its hepatic and intestinal target genes transcriptionally regulate BA synthesis, detoxification, secretion, and absorption in the enterohepatic circulation. Activation of FXR protects from BA overload and liver damage by suppression of BA de novo synthesis, reduction in hepatic uptake of BAs, as well as through stimulation of secretion across the canalicular and basolateral hepatocyte membranes. These mechanisms made FXR and its signaling pathways attractive targets for the treatment of cholestatic liver diseases. While the first in class FXR agonist obeticholic acid has already been approved for the treatment of PBC in patients with insufficient response toward UDCA or with UDCA intolerance, further nonsteroidal and more selective FXR modulators are being developed. These agents may avoid prolonged systemic activation of FXR, allow for a tissue-specific targeting, and also dissociate different FXR signaling effects. The first of these nonsteroidal FXR agonists have progressed into clinical phase II trials in PBC (tropifexor). In PSC both OCA and cilofexor have reached clinical phase II trials. Furthermore, the nontumorigenic FGF19 analogue NGM282 has been tested in patients with PBC as well as PSC with favorable results. Thus, over the next years, more agents targeting the FXR-FGF19 pathway will progress into phase III trials to determine efficacy, safety, and tolerability, and the future will show whether the results seen in the phase II trials

translate into clinically meaningful outcome improvements for patients with chole-static liver diseases.

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