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Stefano Fiorucci
Eleonora Distrutti *Editors*

Bile Acids and Their Receptors

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Stefano Fiorucci • Eleonora Distrutti
Editors

Bile Acids and Their Receptors

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Editors
Stefano Fiorucci
University of Perugia
School of Medicine
Perugia, Italy

Eleonora Distrutti
S.C. Gastroenterologia
Azienda Ospedaliera di Perugia
Perugia, Italy

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Preface

The Pharmacology of Bile Acid-Activated Receptors: A Successful History

Bile acids are the end product of cholesterol metabolism in mammals known for their role in nutrient absorption. Bile acids are amphipathic steroids, acting as physiological surfactants that thanks to their molecular arrangement develop a peculiar hydrophilic/lipophilic balance in aqueous solutions facilitating the absorption of dietary fats (lipids and cholesterol) and fat-soluble vitamins through micellar dispersion. In the last 20 years, however, there has been a dramatic increase in the extent of our knowledge regarding the functional role of bile acids driven by the discovery that, similar to other steroids, bile acids are signaling molecules acting on a specific family of receptors highly represented in the gastrointestinal tract and liver.

The first bile acid receptor, a nuclear receptor, was identified in 1999, when the farnesoid X receptor (FXR) was *deorphanized* and demonstrated to be the receptor for chenodeoxycholic acid (CDCA) and cholic acid (CA), two primary bile acids in humans. Since then, other nuclear receptors, namely the pregnane X receptor (PXR), the constitutive androstane receptor (CAR), the vitamin D receptor (VDR), and the liver X receptors α and β (LXR), have been shown to function as receptors for bile acids. In contrast to FXR, the later receptors, however, can be activated also by other steroids and intermediates in the cholesterol biosynthetic pathways, and therefore FXR remains the most specific bile acid sensor. Importantly, these receptors are widely expressed throughout the mammalians body, and this discovery has greatly extended our understanding of the role of bile acids in physiological and pathological settings.

In 2002, it was discovered that secondary bile acids, such as lithocholic acid (LCA) and deoxycholic acid (DCA), can activate a totally different family of receptors, belonging to G-protein-coupled receptor (GPCR) family. The first GPCR identified as bile acid-activated receptor was named as M-BAR (membrane bile acid-activated receptor) or TGR5 (Takeda G protein receptor 5) and then christened as GPBAR1 (IUPHAR/BSP – www.guidetopharmacology.org). Shortly, it was shown that this receptor exerts an important role in regulating a key aspect of glucose and lipid metabolism and energy expenditure. Later, other GPCRs, such as the sphingosine-1-phosphate receptor 2 (S1P-R2) and the cholinergic receptors,

have shown to be activated by primary and secondary bile acids. Together, these receptors represent a novel, though heterogeneous family of receptors: the so-called bile acid-activated receptors (BARs).

The molecular biology of BARs is complex, and some of the nuclear receptors included in this family are per se potent and pleiotropic regulatory factors. An example is FXR, a master gene, that functions as an heterodimer with the retinoid X receptor (RXR) and whose activation regulates the expression/activity of a number of downstream genes involved in the regulation of uptake, metabolism, and excretion of bile acids by hepatocytes as well as the uptake of endobiotic and xenobiotic by the intestine and liver, along with other transcription factors directly or through the transcription of other regulatory factors such as the orphan receptor SHP (small heterodimer partners). Further on the activation of these receptors in the intestine triggers the release of potent regulatory factors such as GLP-1 (by GPBAR1) and the fibroblast growth factor 15/19 (by FXR) establishing potent entero-hepatic and entero-insular axes that widen the activity of GPBAR1 and FXR to distant targets.

The main focus of this monography is to present the latest results and advances in the field of bile acids as signaling molecules and how these receptors have become major pharmacological targets. The book covers all major areas of research in this field, from chemistry, in silico modeling, molecular biology to clinical applications, offering a view of the functional role of bile acids as signaling molecules, virtually acting on all major areas of human metabolism. Indeed, while FXR and GPBAR1 are essentially bile acid sensors that integrate the de novo bile acid synthesis, with intestinal microbiota and liver metabolism, in a broader sense, BARs play pathogenic role in the development of common alignments including liver, intestinal, and metabolic disorders, such as steatosis (NAFLD) and steatohepatitis (NASH), diabetes, obesity, and atherosclerosis. Further on, in some tissues, BARs are an essential regulator of normal and neoplastic growth and are required to maintain intestinal immune and metabolic homeostasis.

Following this explosion of our knowledge, BARs have been shown to be potentially *druggable,* and selective and nonselective FXR and GPBAR1 ligands, of steroidal and nonsteroidal structure, and have recently entered the therapeutic armamentarium. Of interest, even FXR antagonists have recently shown to have a therapeutic potential.

We hope that the book will be of interest to a wide readership including basic and clinical researchers and scholars/academic in gastroenterology, endocrinology, immunology, medicine, and surgery. General readers and students will find a fascinating description on how science leads drug discovery, and in a few years can move from innovative concept to patient treatment.

This book represents the results of the coordinated effort of several investigators. Some of the authors have done seminal works in the field, indeed most of them have settled the field, and we are proud to have been able to gather all together.

As per any scientific area, the bile acid field is a work in progress. We have made an effort to make this book a useful tool for beginners and experts and hope to have succeeded in the task.

We wish to thank all the authors for their contributions.

A special thanks goes to Prof. Pierangelo Geppetti and the Springer staff.

Perugia, Italy Stefano Fiorucci
Perugia, Italy Eleonora Distrutti

Contents

The Pharmacology of Bile Acids and Their Receptors

Stefano Fiorucci and Eleonora Distrutti

Contents

Abstract

This review provides a historical perspective of bile acids and their receptors as therapeutic targets. Bile acids are atypical steroids generated by the liver from cholesterol and have been used for almost half a century for treating liver and biliary disorders. Since the early 1970s of the last century, chenodeoxycholic acid (CDCA), a primary bile acid, and ursodeoxycholic acid (UDCA), a secondary bile acid and the 7βepimer of CDCA, have been shown effective in promoting the dissolution of cholesterol gallstones. However, lack of activity and side effects associated with the use of CDCA, along with the advent of laparoscopic

S. Fiorucci (✉)
Section of Gastroenterology, Department of Surgical and Biomedical Sciences, University of Perugia, Perugia, Italy
e-mail: Stefano.fiorucci@unipg.it; http://www.gastroenterologia.unipg.it/

E. Distrutti
Azienda Ospedaliera di Perugia, Perugia, Italy

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cholecystectomy, have greatly reduced the clinical relevance of this application. At the turn of the century, however, the discovery that bile acids activate specific receptors, along with the discovery that those receptors are placed at the interface of the host and intestinal microbiota regulating physiologically relevant enterohepatic and entero-pancreatic axes, has led to a "bile acid renaissance." Similarly to other steroids, bile acids bind and activate both cell surface and nuclear receptors, including the bile acid sensor farnesoid X receptor (FXR) and a G-protein-coupled bile acid receptor, known as GPBAR1 (TGR5). Both receptors have been proved druggable, and several highly potent, selective, and nonselective ligands for the two receptors have been discovered in the last two decades. Currently, in addition to obeticholic acid, a semisynthetic derivative of CDCA and the first in class of FXR ligands approved for clinical use, either selective or dual FXR and GPBAR1 ligands, have been developed, and some of them are undergoing pre-approval trials. The effects of FXR and GPBAR1 ligands in different therapeutic area are reviewed.

Keywords
Bile acids · FXR · Glucose metabolism · GPBAR1 · G-protein-coupled receptors · Lipid metabolism · Liver · Nuclear receptors

1 Introduction

Bile acids are a family of steroidal molecules generated in the liver from cholesterol breakdown. Because of the atypical configuration of the A/B junction, bile acids are unable to bind conventional steroidal receptors. In contrast, however, they activate a family of specific receptors known as the "bile acid-activated receptors" (BAR) that are unresponsive to conventional steroids such as estrogen, androgen, and glucocorticoids (Table 1).

2 Bile Acids, a Family of Unconventional Steroids

The human liver metabolizes cholesterol to generate two major bile acids, known as primary bile acids, that are then transported through the bile ducts to the intestine, where they undergo a series of modifications operated by members of intestinal microbiota, to generate the so-called "secondary" or "degenerated" bile acids (Figs. 1 and 2). Thus the variety of bile acid species found in mammalians result from the coordinated cooperation of human and bacterial genes, and secondary bile acids are bacterial products involved in regulation of host metabolism.

Primary bile acids are synthesized by liver cells, the hepatocytes, by the coordinated intervention of at least 17 enzymes (Li and Chiang 2014), which are grouped

Table 1 The family of bile acid-activated receptors

Receptor	Tissue distribution	Natural bile acid agonists Rank of potency	Synthetic ligands under evaluation for potential clinical applications
Cell membrane receptors			
GPBAR1 (TGR5)	Ileum, macrophages, gallbladder, adipose tissues	LCA>DCA>CDCA>UDCA>CA	INT-767, INT-777, BAR501, BAR502
Sphingosine-1-phosphate receptor 2 (S1PR2)	Hepatocytes	GCA,TCA, GCDCA, TCDCA, GDCA and TDCA	
Muscarinic receptor M2 and M3	CNS, smooth muscle cells	DCA-LCA	
Formyl peptide receptor 1 (FMLP)	Macrophages	CDCA (antagonist)	
Vascular endothelial growth factor (VEGF)-R	Gastric and colon cancer cell lines	CDCA	
Nuclear receptor			
FXR (NR1H4)	Hepatocytes, small intestine, macrophages NKT cells, adipocytes	Agonists: CDCA>CA>LCA>DCA Antagonists: βMuricholic acid (mouse)	GW4064, Obeticholic acid (6-ECDCA), BAR502, fexaramine, Px-104, tropifexor (LJN452), LMB763
LXRαβ	Hepatocytes	Hyo-DCA	
VDR (NR1H1)	Ileum, macrophages, endocrine tissues, skin	LCA	
PXR (NR1H2	Hepatocytes	3-keto-LCA, LCA, CDCA DCA, CA 7-hydroxy-cholesten-3-one	
CAR (NR1H3)	Hepatocytes	CA, 6-keto-LCA	

into two major metabolic pathways: the classical and alternative (also known as "acidic") pathways (Fig. 2).

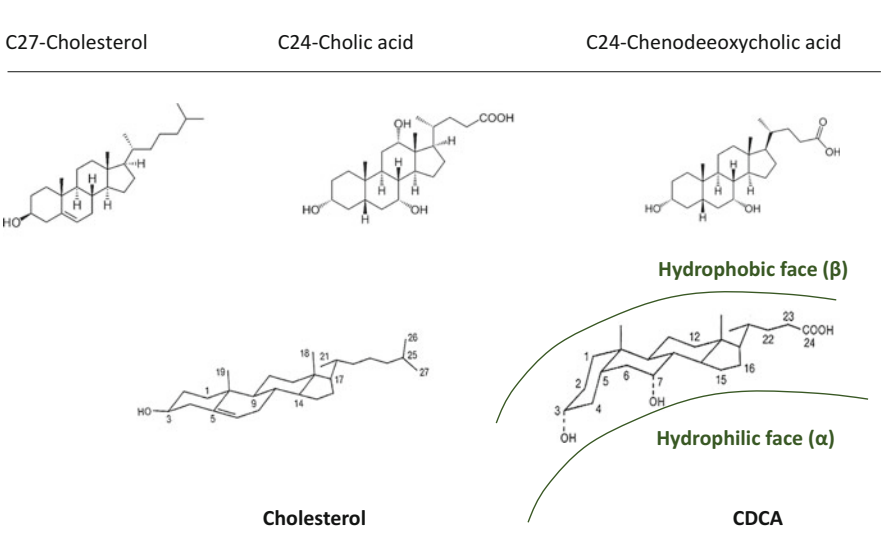

Fig. 1 Upper panel. Structure of cholesterol, cholic acid, and chenodeoxycholic acid. Lower panel. Structure of cholesterol and CDCA. The disposition of the OH groups at positions 3 and 7 directed toward the α-face explains the amphipathic properties of CDCA in comparison to cholesterol, creating a hydrophobic β face and a hydrophilic α face

In the classical pathway, which takes place only in the liver, the first, and rate-limiting enzyme, in bile acid synthesis is the cholesterol 7α-hydroxylase (CYP7A1), which generates a metabolite, the 7α-hydroxycholesterol, which is then converted into the 7α-hydroxy-4-cholesten-3-one, an intermediate metabolite that could be used for generating both CA and CDCA (Fig. 2). In the alternative pathway, the cholesterol breakdown starts with the oxidation of the side chain. The first enzyme involved in this pathway is the sterol 27-hydroxylase (CYP27A1) that generates the 27-hydroxycholesterol. After these initial steps, the two pathways converge, since CYP27A1 is required for the side chain oxidation in both pathways. The classic pathway contributes CA and CDCA in almost similar quantities and is responsible for the production of the large majority of the bile acid pool in normal settings (~90%), while the alternative pathway only generates CDCA and contributes less than 10% of total bile acid pool (Li and Chiang 2014; Fiorucci and Distrutti 2019).

Bile acid biosynthesis in the liver introduces several changes to the structure of cholesterol (Fig. 1). In fact, in contrast to cholesterol, bile acids are amphipathic molecules (Fig. 1), endowed with a hydrophobic side (β face) and a hydrophilic side (α face). The amphipathic nature of bile acids is essential for solubilizing lipids in the

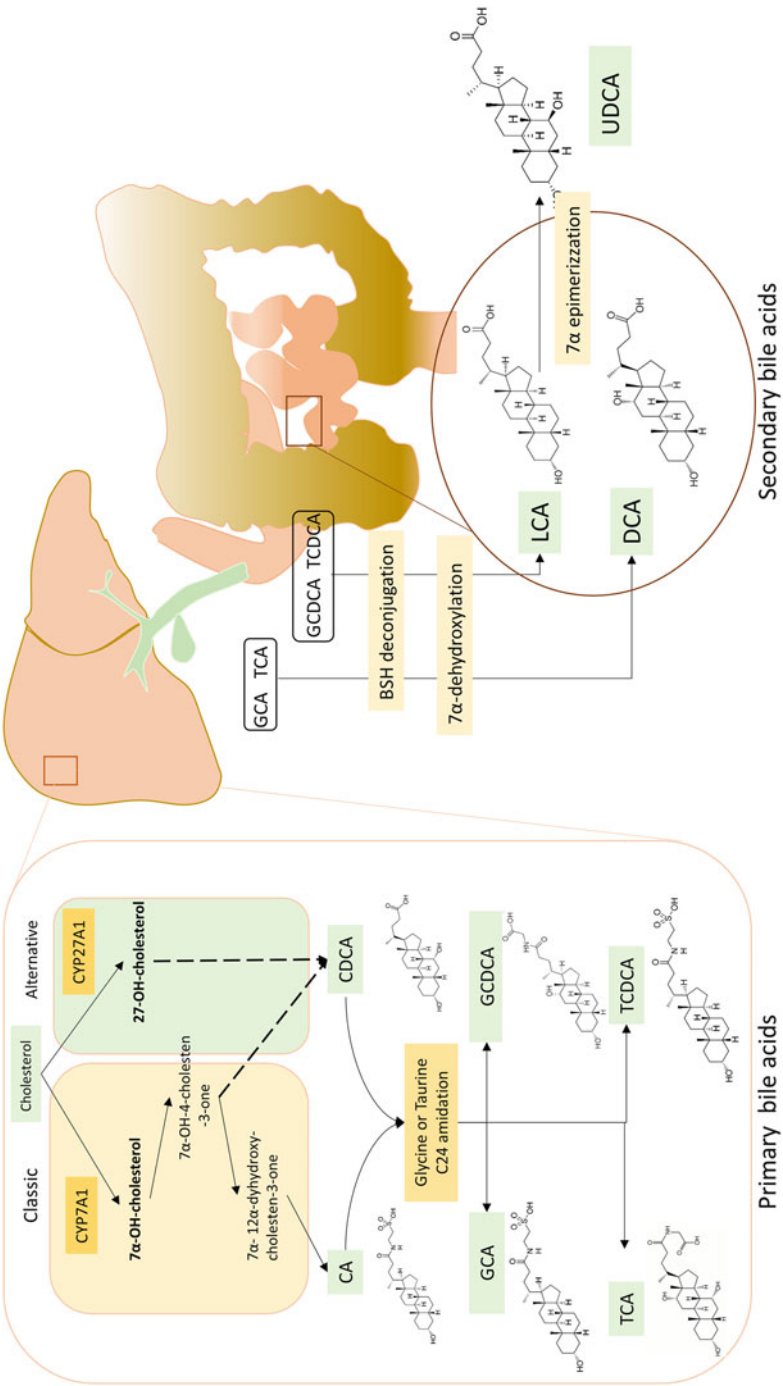

Fig. 2 Bile acid synthesis by hepatocytes. Bile acid are synthesized in the liver from cholesterol by two major pathways, known as the "classical" and the "alternative" pathways. In the classical pathway, the first and rate-limiting enzyme is the CYP7A1, while the alternative pathway begins with reduction of the side chain of cholesterol by the CYP27A1. The two pathways converge to generate the two main primary bile acids in humans, i.e., cholic acid (CA) and

form of mixed micelles (Hofmann and Roda 1984) (Fig. 1). The amphipathic properties of bile acids are due to the unique stereochemistry of the A/B ring juncture that is caused by the reduction of the double bond in B ring of cholesterol to give a 5β (A/B *cis*) A/B ring juncture. The reduction of the double bond in the B ring, however, is only one of the biotransformations of cholesterol that take place in the hepatocytes. Additional changes are (1) the β-oxidation of the side chain of cholesterol that allow the removal of three carbon atoms (from C27 to C23), (2) the conversion of the terminal carbon of cholesterol in to a carboxyl group. (3) the α-hydroxylation at the positions 7 and 12, and (4) the epimerization of the 3-β-hydroxy group to a 3α-hydroxy group.

These changes (Figs. 1 and 2) lead to generation of a 3α- and 7α-dy-hydroxylated bile acid, i.e., the chenodeoxycholic acid (CDCA) [3α, 7α-dihydroxy-5β-cholan-24-oic acid], and the 3α, 7α, 12α tri-hydroxylated bile acid, i.e., the cholic acid (CA) [3α, 7α, 12α-trihydroxy-5β-cholan-24-oic acid] (Li and Chiang 2014). CDCA and CA are the two main primary bile acids in humans. Before their excretion into the canalicular duct, bile acids are amidated (i.e., conjugated) with glycine and taurine, by hepatocytes and, to lesser extent, by cholangiocytes. These amidated derivatives, i.e., glyco-CA and glyco-CDCA (GCA and GCDCA) and tauro-CA and tauro-CDCA (TCA and TCDCA), are indicated as bile salts and are secreted into the bile, and transported to the intestine, where they undergo a series of biotransformations mediated by the intestinal microbiota. Specific bacterial enzymes operate first the deamidation of bile salts (i.e., the removal of glycine and taurine), to generate non-conjugated primary bile acids, and then the 7-dehydroxylation, oxido-reduction, epimerization, and side chain desaturation to generate the secondary bile acids (Figs. 1 and 2). The final results of these bacterial activities in the human intestine are three secondary bile acids, the lithocholic acid (LCA), deoxycholic acid (DCA), and ursodeoxycholic acid (UDCA) that is the 7β-epimer of CDCA (Fig. 1). Different bile acid species are generated in rodents including the α- and β-muricholic acids (MCA) that are generated from CDCA and are primary bile acids in mice. In mice also UDCA is a primary bile acid. Approximately 95% of these deconjugated primary bile acids and secondary bile acids are reabsorbed in the distal ileum and transported back to the liver through the portal vein, completing a cycle in the so-called enterohepatic circulation. Only LCA that is formed in the colon from CDCA is poorly reabsorbed and excreted with the feces.

Fig. 2 (continued) chenodeoxycholic acid (CDCA). CA and CDCA are then amidated with glycine or taurine in the liver, to form the bile salts (GCA and GCDCA, and TCA and TCDCA), and then released into the intestine. In the small intestine, CA and CDCA are first deamidated and deydroxylated to generate DCA and LCA, i.e., the secondary bile acids. The 7β epimerization of CDCA leads to the formation of ursodeoxycholic acid, UDCA, which is a secondary bile acids in human. *CDCA* chenodeoxycholic acid, *DCA* deoxycholic acid, *LCA* lithocholic acid, *CA* cholic acid, *TLCA* taurolithocholic acid, *UDCA* ursodeoxycholic acid, *GCA* glycocholic acid, *TCA* taurocholic acid, *GDCA* glycodeoxycholic acid, *TDCA* taurodeoxycholic acid, *TUDCA* tauroursodeoxycholic acid

3 Bile Acids Before the Discovery of Their Receptors

Bile acids are the main component of the bile and the driving force that maintains the bile flow, and their relative concentrations are an important factor to maintain cholesterol solubility in the bile. Once released in the intestine, bile acids aggregate dietary lipids in micellae, an essential step for the absorption of dietary lipids and liposoluble vitamins by the terminal ileum. These functional activities are due to the amphipathic property of bile acids, which is measured by their critical micellar concentration (CMC), an indirect measure of their hydrophobicity. The CMC has been used for decades to explain the pharmacological benefits of bile acids: particularly their ability to maintain cholesterol in solution and promote the dissolution of cholesterol stones in the gallbladder (Carey and Small 1978; Danzinger et al. 1972).

Building on this somewhat preliminary knowledge of bile acid physiology, CDCA has been developed into a drug almost 50 years ago. In 1972, two different historical papers were published in *The Lancet* and *The New England Journal of Medicine* showing that oral treatment with CDCA for at least 6 months promoted the dissolution of gallbladder cholesterol stones (Bell et al. 1972; Danzinger et al. 1972). In 1973 CDCA become the first oral treatment approved for gallstone dissolution and was indeed the first bile acid that entered the therapeutic armamentarium. In 1975, Makino et al. reported that also UDCA, the 7β-epimer of CDCA, was effective in promoting gallstone dissolution. Since then, because its enhanced safety and superior efficacy profile in comparison to CDCA, UDCA, at classical doses of 5–15 mg/kg/day, has become the choice therapy for gallstone dissolution and has been widely used for this indication up to the advent of laparoscopic cholecystectomy in the 1990s of last the century. Since then, this giant progress in the surgical technique has resulted in a progressive decline of the interest in gallstone dissolution therapies, greatly reducing the interest in the bile acid pharmacology (Fiorucci and Distrutti 2019; Cabrera et al. 2019).

UDCA, however, has gained its way as a prime-time therapy in the treatment of patients with primary biliary cholangitis (PBC). UDCA has remained the only therapy for PBC patients for many years and has been shown effective on several "hard" endpoints, slowing down the histologic progression, delaying the time for the liver transplantation, and reducing mortality. UDCA has gained approval for the treatment of this disorder, although the mechanism of action of UDCA, beside its choleretic activity, remains elusive (Poupon 2010). Currently, approximately 75–80% of PBC patients are effectively treated with 15–30 mg/kg/day of UDCA (Arrese 2019).

4 The Discovery of the Bile Acid-Activated Receptors: FXR and Other Nuclear Receptors

A giant step in the bile acids history was made in 1999, when three different groups reported that primary bile acids activate a receptor, known as the farnesoid X receptor (FXR) (Makishima et al. 1999; Parks et al. 1999; Wang et al. 1999). FXR was therefore identified as the first bile acid-activated receptor. While FXR is one among several nuclear receptors that were deorphanized at the end of the last century, the discovery that CA and CDCA might function as the natural ligand for a nuclear receptor has generated a renaissance in the bile acid arena.

FXR was originally identified in 1995 as a putative receptor for farnesol, an intermediate in cholesterol synthesis, by Forman et al. (1995) while working at the Salk Institute in San Diego, CA, and is a typical member of the nuclear receptors superfamily (Chawla et al. 2001). FXR binds to its target genes as an heterodimer with the retinoid X receptor (RXR) and therefore belongs to the family 1 of nuclear receptors that includes, beside FXR itself, the retinoid A receptor (RAR), the liver X receptor (LXR) α and β, the pregnenolone X receptor (PXR), the vitamin D receptor (VDR), the constitutive androstane receptor (CAR), the peroxisome proliferator-activated receptors (PPAR)αβγδ, and the thyroid receptor (TR). A selective subgroup of these receptors, which are activated by bile acids, i.e., FXR, CAR, LXRs, PXR, and VDR, are also indicated as bile acid-activated receptors (BARs) (Table 1). Some of these receptors are highly expressed in the liver and regulate major steps of cholesterol metabolism, and endo- and xenobiotics including bile acids, bilirubin, and drugs (Fiorucci et al. 2007; Fiorucci and Distrutti 2015; Swanson et al. 2013). These ligand-regulated transcription factors are also described as "adopted orphan receptors" (Chawla et al. 2001), to separate them from "classic" steroidal receptors and "orphan" nuclear receptors (a family that includes the largest group of nuclear receptors whose physiological ligands have not been identified yet).

4.1 FXR, the Bile Acid Sensor in Drug Discovery: 1999–2019

Immediately after the identification of FXR as the *bona fide* receptor for primary bile acids, this receptor was shown to function as a "bile acid sensor." Indeed, FXR is a master gene that regulates virtually every aspect of the bile acid physiology, i.e., uptake of bile acids by hepatocytes and intestinal cells, liver metabolism and excretion, and kidney metabolism (Fig. 3). Later on, it was shown that FXR also regulates some aspects in lipid (Claudel et al. 2002; Watanabe et al. 2004) and glucose homeostasis (Ma et al. 2006) and insulin secretion by pancreatic β cells (Renga et al. 2010). Some of the effects exerted by FXR are mediated by the direct binding of the FXR/RXR heterodimer to FXR-responsive elements (FXR-RE) in the promoter of target genes (reviewed by Fiorucci et al. 2007); others are mediated by

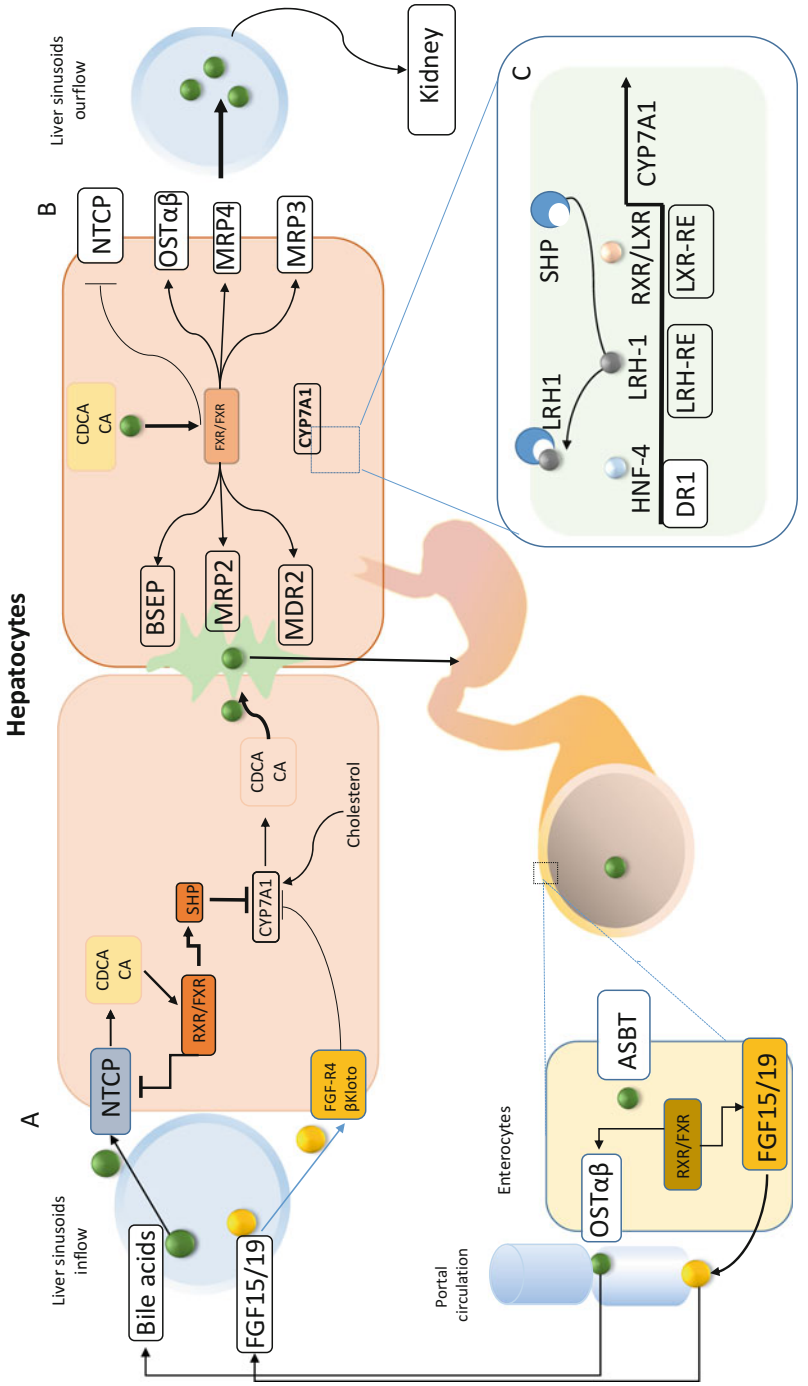

Fig. 3 Regulation of bile acid synthesis and secretion in hepatocytes by FXR. FXR is expressed in liver cells, and upon binding to RXR, the FXR/RXR heterodimer is recruited to a FXR-RE in the promoter of target genes. In the setting of high intracellular concentrations of bile acids, FXR become activated and

the regulation of other transcription factors such as the nuclear receptor SHP (small heterodimer partner), an atypical nuclear receptor that lacks the DNA-binding domain (Seol et al. 1996). While recruitment of FXR to the FXR-RE in target genes promotes their transcription, SHP acts in most circumstances as a gene repressor (Fig. 3), terminating the activity of key genes involved in bile acid synthesis such as CYP7A1l (Fig. 3), highlighting a sophisticated mechanism of regulation of bile acid synthesis by FXR (Fig. 3). FXR integrates the endogenous synthesis of bile acids with the enterohepatic circulation (Goodwin et al. 2000). Not surprisingly these seminal works have led to the discovery of novel therapeutic targets including entero-liver and entero-metabolic axis and shown that FXR regulates the synthesis and release of a member of the fibroblast growth factor family, FGF-15 (19 is human ortholog). Once released by ileal enterocytes, FGF15 reaches the liver traveling the portal circulation. At the sinusoidal pole of hepatocytes (Fig. 3), FGF15/19 binds to the FGF-R4/βkloto complex activating a negative pathway that represses CYP7A1 activity and bile acid synthesis (Inagaki et al. 2005; Moschetta et al. 2019). Further on FGF15/19 causes gallbladder muscle relaxation and is the main player of the so-called enterohepatic axis that negatively regulates the synthesis of endogenous bile acids. FGF15/19 is also effective in increasing insulin sensitivity and is currently exploited in the treatment of major metabolic disorders, including diabetes, obesity, and nonalcoholic steatohepatitis (NASH) (Moschetta et al. 2019).

From a pharmacological stand point, the discovery that FXR increases bile acid flow suggested that this receptor could have been exploited to treat medical conditions linked to the bile flow impairment (cholestasis). While many diseases are characterized by an impaired bile flow, the prototypical disorders are PBC and the primary sclerosing cholangitis (PSC). However, cholestasis might arise from a

Fig. 3 (continued) is recruited to the promoter of SHP. SHP in its turn represses the activity of CYP7A1 the rate-limiting enzyme in the classical pathway of bile acid synthesis. In addition to this SHP-mediated effect, FXR directly represses the expression of NTCP, thus reducing the uptake of bile acid from sinusoids, and increases the transcription of bile acid extruder either at the biliary side of hepatocytes (BSEP, MRP2 and MDR3) and the basolateral side (OSTs, MRP3, MRP4). The aggregate effects of these activities are to reduce uptake and endogenous synthesis of bile acids and to promote excretion from hepatocytes. In addition to these liver effects, FXR releases FGF15/19 from enterocytes of the terminal ileum. FGF15/19 reaches the liver through the portal circulation and after binding to the FGF-R4/βkloto complex on hepatocyte's surface negatively regulates the expression/activity of CYP7A1. Inset C. SHP functions as a negative regulator for CYP7A1. SHP is an atypical nuclear receptor that lacks the DNA-binding domain. SHP binds to LRH1, thus removing this regulatory factor from the promoter of CYP7A1. This associates with loss of co-activators and recruitment of co-repressor, terminating the transcription of CYP7A1. *ASBT* sodium-dependent *bile acid* transporter (SLC10A2), *BSEP* bile salt export pump, *CYP7A1* cholesterol 7α-hydroxylase, *FXR* farnesoid X receptor, *(FGF)15/19* fibroblast growth factor, *FGHF-R4* FGF receptor 4, *HNF4* hepatocyte nuclear factor 4, *LXR* liver X receptor, *LRH-1* liver receptor homolog-1, *MRP2* multidrug resistance-associated protein 2, *MRP3* multidrug resistance-associated protein 3, *MRP4* multidrug resistance-associated protein 4, *NTCP* sodium taurocholate co-transporting polypeptide, *OSTα/β* organic solute transporter alpha and beta, *RXR* retinoic X receptor, *SHP* small heterodimer partner

number of disorders, the most common of which are side effects of drugs or of genetic mutations affecting bile acid transporters some of which are regulated by FXR. While UDCA, as mentioned above, is used for treating these disorders, and particularly PBC, it was found that UDCA per se exerted no effect on FXR, thus opening the possibility to combine UDCA with a FXR ligand. The first synthetic FXR ligand, the GW4064, was originally discovered in 2000 by Maloney et al. (2000) at the GlaxoSmithKline (Research Triangle Park, NC, USA). While over the years, this compound has been extensively used as a tool for its selectivity toward FXR, it turned out that it had a low plasma bioavailability and therefore was never developed into a drug. In 2002, we reported the discovery of a semisynthetic derivative of CDCA, as a relatively potent ligand for FXR (Pellicciari et al. 2002). This agent was named the 6-ethyl-CDCA by its chemical structure and then renamed as INT-747 in 2004 and later christened as obeticholic acid (Fiorucci and Distrutti 2019). In 2016, obeticholic acid has gained approval for the treatment of UDCA-resistant PBC patients and is currently undergoing pre-approval trials for the treatment of NASH. Following these progresses, many other selective FXR ligands of steroidal and nonsteroidal nature have been developed (De Marino et al. 2019), and some of them are undergoing clinical trials. In 2015, we have reported a new generation of steroidal non-bile acid selective FXR ligand endowed with a potent anti-inflammatory and lipid-lowering activity (Sepe et al. 2018).

5 Secondary Bile Acids and GPBAR1

While primary bile acids activate FXR, the secondary bile acids, DCA and LCA, are the physiologic ligands for a G-protein-coupled receptor known as M-BAR or Takeda G-protein receptor (GR)5 discovered in 2002 by Maruyama et al. (2002). TGR5 has since then renamed as G-protein bile acid-activated receptor 1 (GPBAR1) (IUPHAR-BJP nomenclature). High levels of GPBAR1 mRNA were detected in several organs such as the small intestine, stomach, liver, lung, placenta, and spleen. The receptor, however, is not expressed by the liver parenchymal cells, hepatocytes, while non-parenchymal cells, such as liver sinusoidal cells and Kuppfer cells, the liver resident macrophages, express the receptors as shown by Keitel et al. in 2007 (Keitel et al. 2007; Keitel 2019). GPBAR1 plays important roles in cell signaling, and its ligation by DCA and LCA increases cAMP concentrations and regulates the expression of several genes in target cells by increasing the recruitment of cAMP response element-binding protein (CREB) to CRE (cAMP response elements) in the target genes (Fig. 4). Additionally, it has been shown that GPBAR1 negatively regulates the phosphorylation of nuclear factor κB (NF-κB) while increases the phosphorylation of AKT and extracellular signal-regulated kinases (ERK) 1 and 2 (reviewed by Fiorucci et al. 2018). A major finding in GPBAR1 physiology has been the demonstration that it might have a role in regulating energy expenditure. In adipocytes and muscle cells, activation of TGR5 (GPBAR1) increases the level of cAMP-dependent thyroid hormone-activating enzyme, the type 2 iodothyronine deiodinase (D2). D2 is a major thermogenic protein that converts thyroxine

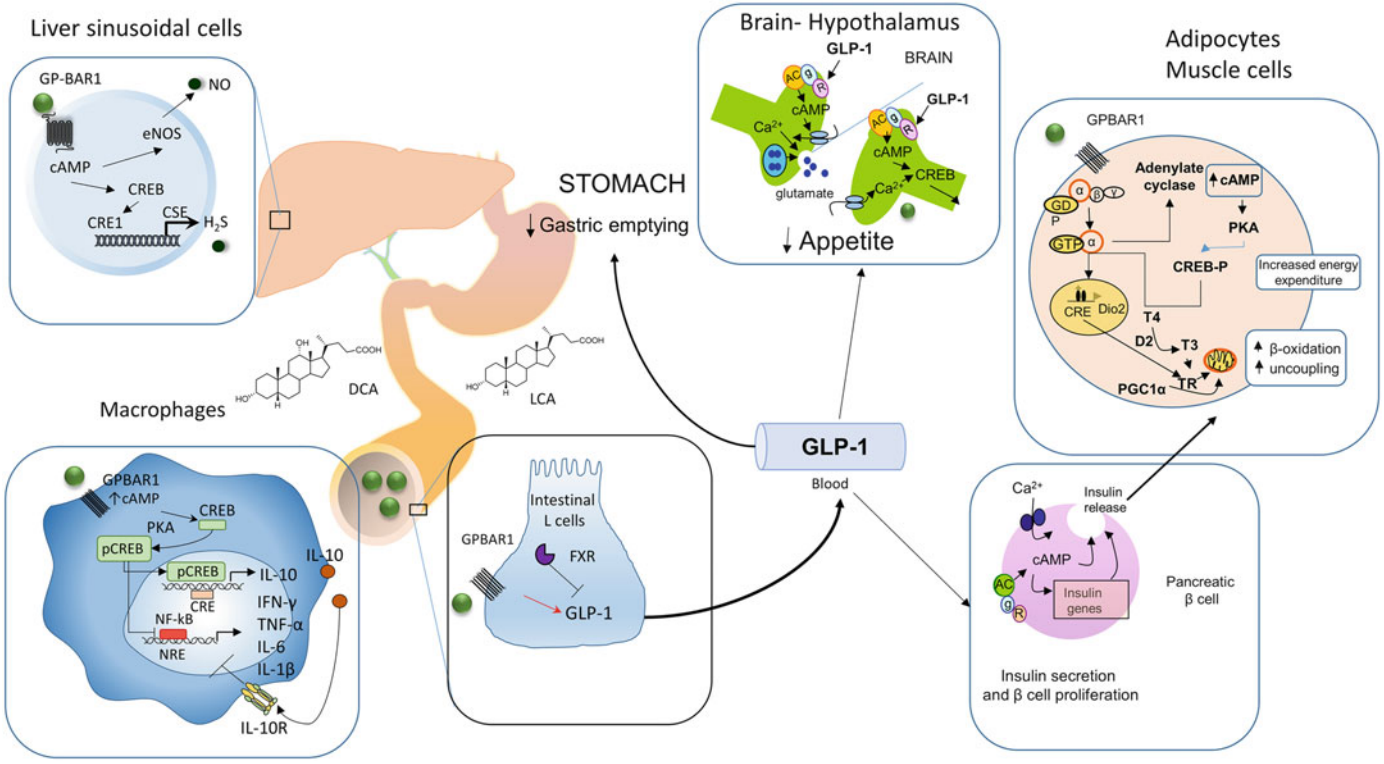

Fig. 4 Physiological effects of GPBAR1. The GPBAR1 is a receptor for secondary bile acids, DCA and LCA, expressed in several tissues. Under ligation, GPBAR1 causes the release of GLP-1 from the L cells located in terminal ileum. Several effects of GPBAR1 ligation on insulin sensitivity are therefore due to GLP-1. In the adipocytes and muscles, GPBAR1 promotes the conversion of T4 in T3 and un-coupling of the mitochondrial respiratory chain, leading to energy dissipation. GPBAR1 is also a negative regulator of inflammation in macrophages and promotes nitric oxide (NO) and hydrogen sulfide (H_2S) formation by endothelial cells, exerting vasodilatory effects. *CREB* cAMP response element-binding protein, *CRE* cAMP response elements, *eNOS* endothelial NO synthase, *CSE* cystathionine-γ-liase, *H2S* hydrogen sulfide

(T4) into the active tri-iodothyronine (T3) in the brown adipose tissue (BAT) and muscles (Fig. 4). Exposure of BAT and human skeletal muscle cells to bile acids increases D2 activity, oxygen consumption, and energy expenditure, suggesting that GPBAR1 could be an anti-obesogenic receptor (Watanabe et al. 2006). In the intestine, GPBAR1 is expressed by L cells, a subtype of entero-endocrine cells that produce the glucagon-like peptide-1 (GLP-1). Activation of GPBAR1 in L cells increases the transcription of the pre-pro-glucagon gene and causes the secretion of GLP-1, an incretin that potentiates postprandial insulin secretion (Katsuma et al. 2005). Activation of GPBAR1 also induces the release neuropeptide hormone peptide tyrosine tyrosine (PYY), which regulates immune signaling and intestinal motility. Together, these data suggest a potential application for this receptor in treating various metabolic diseases including type 2 diabetes, metabolic syndrome, and obesity (Fig. 4). GPBAR1 is also considered a mediator of pruritus and analgesia (Alemi et al. 2013).

A growing area of interest in GPBAR1 pharmacology is linked to its immune-regulatory effects. GPBAR1 is expressed by cells of innate immunity, macrophages, and NKT cells and is deemed essential to maintain intestinal and liver immune homeostasis (Cipriani et al. 2011; Biagioli et al. 2017; Fiorucci et al. 2018).

Several natural and synthetic ligands for GPBAR1 are currently available (Table 1), but so far none has reached the stage of clinical development. However, in addition to selective GPBAR1 ligands, dual FXR/GPBAR1 ligands are currently investigated for their potential in the treatment of highly prevalent human diseases. We have recently described a potent FXR and GPBAR1 ligand, BAR502 (Carino et al. 2017), which has successfully completed preclinical toxicology and is entering a Phase I trial this year. As shown in Fig. 5, FXR and GPBAR1 exert separate but also overlapping effects, making dual FXR/GPBAR1 agonist a valuable approach for the treatment of immune/metabolic disorders.

6 Conclusions

In this chapter we have provided a short overview of the history of therapeutic applications of bile acid and their receptors. Primary and secondary bile acids, CDCA and UDCA, have been used for almost a half century for treating liver and biliary disorders; however, after the discovery that bile acids activate specific receptors, several semisynthetic and synthetic ligands for FXR have been discovered. Currently, in addition to obeticholic acid, the first in class of FXR ligands, either selective or dual FXR/GPBAR1 ligands have been developed, and some of them have completed preclinical toxicology or have been advanced to the therapeutic arena.

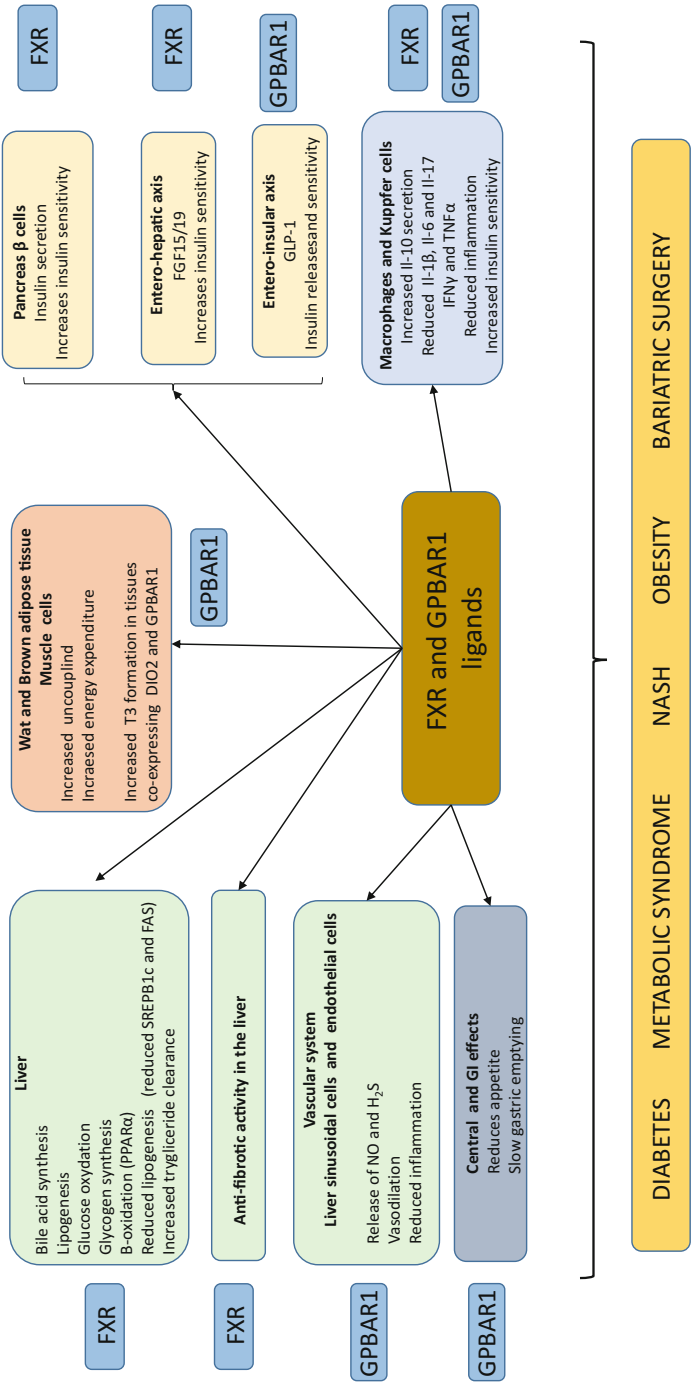

Fig. 5 Pharmacological activities of FXR and GPBAR1 ligands. The key functions of the two receptors on essential step of lipid and glucose metabolism and inflammation are shown

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Bile Acid-Activated Receptors: GPBAR1 (TGR5) and Other G Protein-Coupled Receptors

Verena Keitel, Jan Stindt, and Dieter Häussinger

Contents

Abstract

The BA-responsive GPCRs S1PR2 and TGR5 are almost ubiquitously expressed in human and rodent tissues. In the liver, S1PR2 is expressed in all cell types, while TGR5 is predominately found in non-parenchymal cells. In contrast to S1PR2, which is mainly activated by conjugated bile acids (BAs), all BAs serve as ligands for TGR5 irrespective of their conjugation state and substitution pattern.

Mice with targeted deletion of either S1PR2 or TGR5 are viable and develop no overt phenotype. In liver injury models, S1PR2 exerts pro-inflammatory and

V. Keitel (✉) · J. Stindt · D. Häussinger
Clinic for Gastroenterology, Hepatology and Infectious Diseases, University Hospital Düsseldorf, Medical Faculty at Heinrich-Heine-University, Düsseldorf, Germany
e-mail: Verena.Keitel@med.uni-duesseldorf.de

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pro-fibrotic effects and thus aggravates liver damage, while TGR5 mediates anti-inflammatory, anti-cholestatic, and anti-fibrotic effects. Thus, inhibitors of S1PR2 signaling and agonists for TGR5 have been employed to attenuate liver injury in rodent models for cholestasis, nonalcoholic steatohepatitis, and fibrosis/cirrhosis.

In biliary epithelial cells, both receptors activate a similar signaling cascade resulting in ERK1/2 phosphorylation and cell proliferation. Overexpression of both S1PR2 and TGR5 was found in human cholangiocarcinoma tissue as well as in CCA cell lines, where stimulation of both GPCRs resulted in transactivation of the epidermal growth factor receptor and triggered cell proliferation as well as increased cell migration and invasiveness.

This chapter will focus on the function of S1PR2 and TGR5 in different liver cell types and summarizes current knowledge on the role of these receptors in liver disease models.

Keywords
Bile acid receptor · Bile acids · G protein-coupled receptor · Liver disease · S1PR2 · TGR5

1 Introduction

Bile acids (BAs) are potent signaling molecules with pleiotropic effects not only on BA homeostasis but also on glucose, lipid, and energy metabolism as well as on immune functions. Different types of BA receptors and effector molecules allow for a cell type and BA-specific signaling (Häussinger et al. 2012; Copple and Li 2016; Godoy et al. 2013; Keitel and Häussinger 2012; Pols et al. 2011a). These comprise nuclear receptors, which have a DNA-binding domain and act as ligand-activated transcriptions factors, membrane-bound G protein-coupled receptors, as well as further sensing molecules such as ion channels or endomembrane-bound integrins (α5β1) (Häussinger et al. 2012; Copple and Li 2016; Keitel and Häussinger 2012; Pols et al. 2011a; Keitel et al. 2008a; Gohlke et al. 2013; Zhou and Hylemon 2014). The prototype of BA-sensing nuclear receptors is the farnesoid X receptor (FXR, NR1H4), which is activated by chenodeoxycholic acid, deoxycholic acid, as well as lithocholic acid (Makishima et al. 1999; Parks et al. 1999; Wang et al. 1999). Further nuclear BA receptors comprise the pregnane X receptor (PXR, NR1I2) and the vitamin D receptor (VDR, NR1I1), which are only responsive to the secondary BA lithocholic acid (Staudinger et al. 2001; Xie et al. 2001; Gascon-Barre et al. 2003; Han and Chiang 2009). Nuclear BA receptors are discussed in another chapter of this book. BAs can either directly activate or modulate the signaling of several G protein-coupled receptors (GPCRs). The prototype of GPCRs responsive to BAs is the Takeda G protein-coupled receptor 5 (TGR5, also known as G protein-coupled BA receptor 1 (GPBAR1)), which can be activated by unconjugated and conjugated primary and secondary human BAs (Häussinger et al. 2012; Keitel and Häussinger 2012; Gascon-Barre et al. 2003; Han and Chiang 2009; Kawamata et al. 2003; Keitel

and Häussinger 2018; Sato et al. 2008). Taurine- and glycine-conjugated BAs are also ligands for the sphingosine-1-phosphate receptor 2 (S1PR2, also known as EDG-5) (Zhou and Hylemon 2014; Studer et al. 2012). Furthermore, BAs can modulate the signaling of different types of muscarinic (acetylcholine) receptors (e.g., M2 and M3 receptors) as well as formyl-peptide receptors (FPR) (Cheng et al. 2002; Raufman et al. 2002a, b; Sheikh Abdul Kadir et al. 2010; Chen et al. 2000; Ferrari et al. 2006). Taurine-conjugated ursodeoxycholic acid, which is the first-line treatment for patients with chronic cholestatic diseases, such as primary biliary cholangitis (PBC), activates α5β1 integrins intracellularly on endomembranes (Gohlke et al. 2013; Häussinger et al. 2003; Sommerfeld et al. 2015). Some ion channels and kinase signaling pathways are also responsive to different BAs; however the molecular mechanism by which these sensors are activated or their signaling is modulated by BAs is unknown (Häussinger et al. 2012; Becker et al. 2007a, b; Graf et al. 2002; Reinehr et al. 2004, 2005). This chapter will focus on the role of the GPCRs TGR5 (GPBAR1) and S1PR2 in the liver under physiological conditions and in disease states. Endogenous and synthetic ligands as well as ligand binding to these receptors will be discussed in another chapter of this book.

2 TGR5 and S1PR2 Signaling Pathways

S1PR2 and to a lesser extent TGR5 can couple to different heterotrimeric G proteins (Kawamata et al. 2003; Hong et al. 2010; Kwong et al. 2015; Masyuk et al. 2013; Maruyama et al. 2002; Adada et al. 2013). TGR5 couples in most cell types to a stimulatory G alpha protein ($G\alpha_s$), and thus ligand binding to the receptor triggers activation of adenylate cyclase and elevation of intracellular cyclic AMP (cAMP) levels (Kawamata et al. 2003; Maruyama et al. 2002). However, in cholangiocytes TGR5 can associate with either a $G\alpha_s$ or an inhibitory G alpha protein ($G\alpha_i$) depending on the subcellular localization of the receptor itself (Masyuk et al. 2013). When present in the primary cilium of cholangiocytes, TGR5 couples to $G\alpha_i$ and inhibits cell proliferation (Masyuk et al. 2013). In contrast, when present in the apical plasma membrane, TGR5 associates with $G\alpha_s$ and promotes cell proliferation (Masyuk et al. 2013). Moreover, in a cell line derived from esophageal Barrett's adenocarcinoma (FLO), TGR5 was associated with both $G\alpha_q$ and $G\alpha_{i3}$; however, signal transduction after ligand binding was observed only in coupling with $G\alpha_q$ (Hong et al. 2010). S1PR2 can associate with $G\alpha_i$, $G\alpha_q$, and $G\alpha_{12/13}$ depending on cell type and has been demonstrated to functionally couple to $G\alpha_i$ in hepatocytes (Zhou and Hylemon 2014; Studer et al. 2012; Adada et al. 2013).

TGR5 can be activated by a wide range of ligands, including all known BAs as well as many steroids, such as pregnanolone, allopregnanolone, pregnanediol, and estradiol (Sato et al. 2008; Martin et al. 2013; Keitel et al. 2010a). In contrast to S1PR2, TGR5 recognizes BAs regardless of their substitution and conjugation state (Sato et al. 2008). Ligand binding to TGR5 can activate different kinase pathways

such as protein kinase A (PKA), protein kinase B (AKT), Src kinase, Rho kinase, mammalian target of rapamycin complex 1 (mTORC1), and extracellular signal-regulated kinase 1/2 (ERK1/2) (Kawamata et al. 2003; Masyuk et al. 2013; Lavoie et al. 2010; Perino et al. 2014; Rajagopal et al. 2013; Reich et al. 2016). Moreover, stimulation of TGR5 impairs nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling, triggers a rise in intracellular calcium and reactive oxygen species (ROS) levels, activates different ion channels, modifies gene expression, and promotes mitochondrial fission (Keitel et al. 2007, 2008b, 2009, 2010a; Lavoie et al. 2010; Perino et al. 2014; Reich et al. 2016; Pols et al. 2011b; Thomas et al. 2009; Watanabe et al. 2006; Lieu et al. 2014; Velazquez-Villegas et al. 2018).

Similar to TGR5, ligand binding to S1PR2 can activate multiple downstream signaling pathways (Nagahashi et al. 2016). These include transactivation of the epidermal growth factor receptor (EGFR) and the insulin receptor (IR) resulting in subsequent stimulation of ERK, AKT, and/or c-Jun N-terminal kinase (JNK1/2) (Copple and Li 2016; Studer et al. 2012; Liu et al. 2015). Whether ERK1/2 and AKT can be directly activated by S1PR2 is unclear (Studer et al. 2012). Phosphorylated ERK1/2 may trigger activation, nuclear translocation of NF-κB, and transactivation of NF-κB target genes such as cyclooxygenase-2 (COX-2), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α) (Liu et al. 2015). Moreover, ligand activation of S1PR2 promotes upregulation of sphingosine kinase 2 (SphK2) within the nucleus which in turn increases the synthesis of sphingosine-1-phosphate (S1P). Elevation of nuclear S1P inhibits the function of different histone deacetylases (HDACs) resulting in upregulation of genes encoding nuclear receptors such as FXR and the small heterodimer partner (SHP), the gene encoding the rate-limiting enzyme of BA synthesis CYP7A1 as well as the gene encoding apolipoprotein B-100 (ApoB-100) (Nagahashi et al. 2015).

3 Tissue Distribution of TGR5 and S1PR2

TGR5 and S1PR2 mRNA are expressed almost ubiquitously in rodent and human tissues (Kawamata et al. 2003; Adada et al. 2013; Maruyama et al. 2006; Vassileva et al. 2006; Wang et al. 2014). High levels of TGR5 mRNA were detected in the gallbladder, placenta, spleen, lung, liver, intestine, kidney, adrenal glands, adipose tissue, smooth muscle, female reproductive organs, as well as in fetal liver and kidney (Kawamata et al. 2003; Vassileva et al. 2006). Strong S1PR2 mRNA expression was found in the placenta, gallbladder, liver, lung, intestine, heart, adipose tissue, female reproductive organs, brain, and lymph nodes (see also V18.1.proteinatlas.org) (Ishii et al. 2001; Uhlen et al. 2015).

In rodent and human liver, S1PR2 has been detected in liver parenchymal cells (hepatocytes), cholangiocytes, hepatic stellate cells (HSCs), hepatic myofibroblast (MFs), liver sinusoidal endothelial cells (LSECs), and macrophages (Studer et al. 2012; Liu et al. 2011, 2014, 2015; Li et al. 2011a; Ikeda et al. 2004; Hou et al. 2015; Hughes et al. 2008; Zhang et al. 2013). In contrast to S1PR2, TGR5 expression in hepatocytes is negligible when compared to non-parenchymal cells (Keitel and

Häussinger 2012; Studer et al. 2012; Reich et al. 2016, 2017; Keitel et al. 2008b). Using immunofluorescence staining of rat, mouse, and human livers, TGR5 reactivity was detected in liver sinusoidal endothelial cells (LSECs), in liver resident macrophages (Kupffer cells, KCs), and in cholangiocytes (Keitel and Häussinger 2012, 2013, 2018; Reich et al. 2016; Keitel et al. 2007, 2010b). While no TGR5 staining was observed in quiescent hepatic stellate cells (HSCs), the receptor is upregulated during activation and can be detected in myofibroblast-like HSCs in vivo (Keitel and Häussinger 2018; Keitel et al. 2008b; Sawitza et al. 2015).

Furthermore, both TGR5 and S1PR2 have been detected in intestinal epithelial cells, in astrocytes, neurons, and resident immune cells (microglia) of the central nervous system as well as the enteric nervous system (Keitel et al. 2010a; Chen et al. 2017, 2018a; Blaho and Hla 2014; McMillin et al. 2017; Ward et al. 2013; Alemi et al. 2013a; Karababa et al. 2017; Meng and Lee 2009; Poole et al. 2010). TGR5 was also localized in astrocytes and neurons of the peripheral nervous system including dorsal root ganglia and primary sensory neurons (Lieu et al. 2014; Alemi et al. 2013b). In immune cells, TGR5 was found predominately in CD14-positive monocytes and tissue-resident macrophages, while S1PR2 mRNA was detected in B cells, natural killer cells, mast cells, and to a lesser extent in dendritic cells (Kawamata et al. 2003; Keitel et al. 2008b; Blaho and Hla 2014). In the kidney, TGR5 has been localized in glomeruli, tubules, and principal cells of the cortical colleting ducts, while S1PR2 expression was found in mesangial cells (Liu et al. 2012; Wang et al. 2016; Li et al. 2018).

4 Role of S1PR2 Signaling in Hepatocytes

All five sphingosine-1-phosphate receptors (S1PR1–S1PR5) were detected in hepatocytes, with S1PR1 and S1PR2 being the most abundantly expressed ones (Studer et al. 2012; Karimian et al. 2013). Hydrophobic BAs such as glycochenodeoxycholic acid (GCDC) can trigger apoptosis in hepatocytes (Becker et al. 2007a, b; Graf et al. 2002; Reinehr et al. 2003, 2004, 2005; Karimian et al. 2013; Faubion et al. 1999; Sodeman et al. 2000; Hohenester et al. 2010). Activation of S1PR2 can inhibit GCDC-induced hepatocyte apoptosis (Karimian et al. 2013). Conjugated BAs activate S1PR2 in a dose-dependent manner, resulting in increased phosphorylation of ERK1/2 and to lesser extent also of AKT (Studer et al. 2012). While S1P triggered phosphorylation of ERK1/2 and AKT via S1PR2 in nanomolar concentrations, different conjugated BAs were effective at micromolar concentrations (Studer et al. 2012) (Fig. 1). Increased ERK phosphorylation was observed after treatment with TC, GCDC, taurodeoxycholic acid (TDC), glycocholic acid (GC), as well as tauroursodeoxycholic acid (TUDC) (Studer et al. 2012). One of the downstream targets of ERK1/2 is sphingosine kinase 2 (SphK2), which is located in the nucleus and is one of the isoenzymes mediating the formation of sphingosine-1-phosphate (S1P) from sphingosine (Studer et al. 2012) (Fig. 1). S1P has been identified as potent inhibitor of HDAC1 and HDAC2 (Studer et al. 2012; Hait et al. 2009). Thus, activation of SphK2, which is bound to histone

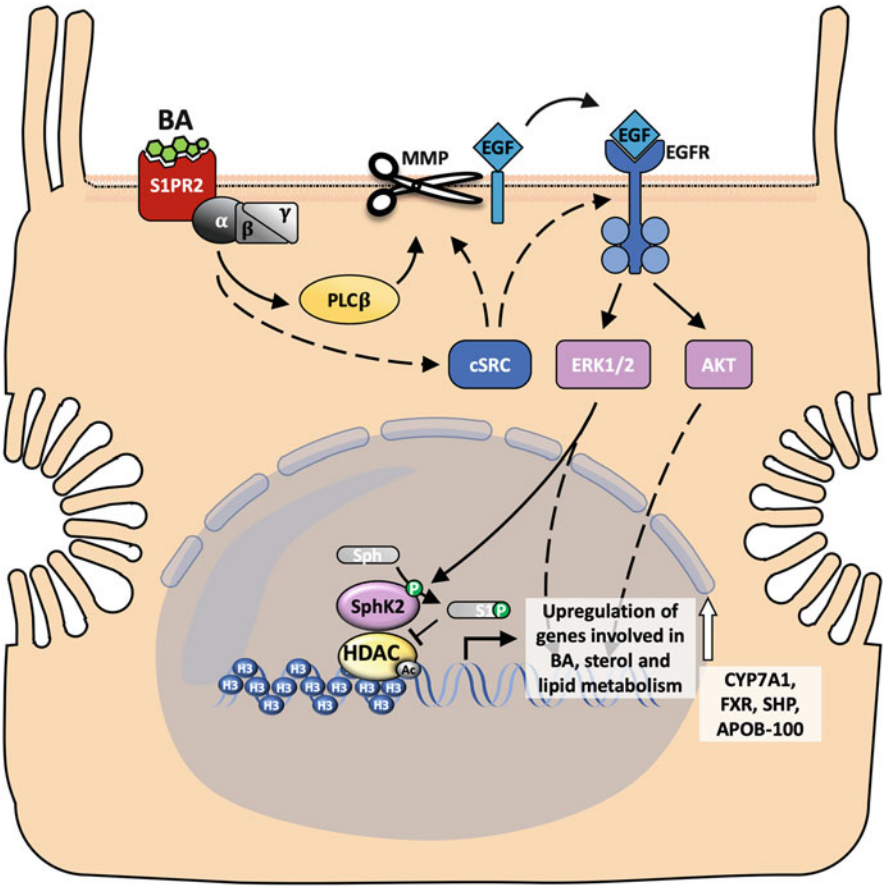

Fig. 1 Role of S1PR2 in hepatocytes. For a detailed description and references refer to text

3 (H3) and associated with specific promotor regions, results in increased S1P levels which subsequently inhibit HDAC1 and HDAC2 promoting H3 acetylation and gene transcription (Studer et al. 2012; Hait et al. 2009). To test whether conjugated BAs can indeed increase SphK2 protein levels and enzyme activity and thus modulate gene expression, TC and the S1PR2 inhibitor JTE-013 were infused into the duodenum of rats for 4 hours prior to harvesting of the livers (Nagahashi et al. 2015). Analysis of hepatocytes revealed an upregulation of SphK2 protein levels and enzyme activity in response to TC, which was abolished by prior JTE3-013 application (Nagahashi et al. 2015). In line with this finding, hepatocytes from mice with targeted deletion of S1PR2 have lower SphK2 mRNA and protein levels as well as enzyme activity, which is unresponsive to TC stimulation (Nagahashi et al. 2015). Overexpression of S1PR2 in murine hepatocytes resulted in increased expression of SphK2 but also of ApoB-100, Shp, Cyp7a1, and Fxr and to a lesser extent also of sterol regulatory element-binding protein 1c (Srebp1c) and of low-density

lipoprotein receptor (LdlR) (Nagahashi et al. 2015). Moreover, liver tissue of SphK2 and S1PR2 knockout (KO) mice contained lower levels of Srebp1c, Fxr, and LdlR mRNA as compared to wild-type animals (Nagahashi et al. 2015). These data suggest that conjugated BAs may modulate sterol and lipid metabolism in the liver via S1PR2, which regulates SphK2 and thus nuclear S1P levels (Nagahashi et al. 2015). Whether the activation of ERK1/2 from S1PR2 is mediated directly or indirectly via transactivation of the EGFR as has been demonstrated for a cholangiocarcinoma (CCA) cell line (HuCCT1) remains elusive to date (Liu et al. 2015) (Fig. 1).

5 Role of S1PR2 and TGR5 in Liver Sinusoidal Endothelial Cells (LSECs)

While little is known on the role of S1PR2 in LSECs, intraperitoneal injection of LPS to wild-type mice resulted in an upregulation of vascular adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1) within sinusoids. This inflammatory response of LSECs was markedly less in S1PR2 KO mice (Zhang et al. 2013). In isolated human umbilical vein endothelial cells (HUVECs), stimulation with TNF-α triggered an S1PR2-dependent upregulation of VCAM-1 and ICAM-1 (Zhang et al. 2013). Ligand binding to S1PR2 via $G\alpha_{12/13}$ coupling resulted in stimulation of the Rho GTPase, Rho-associated protein kinase (ROCK), and NF-κB leading to the upregulation of inflammatory gene expression (Zhang et al. 2013). In parallel, S1PR2 activated p38 stress-activated protein kinase (SAPK), which in turn triggered expression of pro-inflammatory adhesion molecules (Zhang et al. 2013). Inhibition of S1PR2 signaling with JTE-013 prevented monocyte adhesion to TNF-α-stimulated HUVECs (Zhang et al. 2013). Furthermore, S1PR2 activation has been linked to increased vascular paracellular permeability (Sanchez et al. 2007). Thus, S1PR2 plays a role in vascular permeability, inflammatory phenotype, and cell adhesion; however, whether these effects are also induced by conjugated BAs remains elusive (Zhang et al. 2013) (Fig. 2a).

In primary rat LSECs, TGR5 was coupled to $G\alpha_s$ and ligand binding-triggered activation of adenylyl cyclase, subsequent elevation of cyclic AMP, and protein kinase A (PKA)-mediated serine phosphorylation of endothelial nitric oxide synthase (eNOS) resulting in increased generation of nitric oxide (NO) (Keitel and Häussinger 2012, 2018; Reich et al. 2017; Keitel et al. 2007). Stimulation of TGR5 promoted serine phosphorylation of cystathionine γ-lyase (CSE) in an AKT-dependent manner, resulting in increased levels of hydrogen sulfide (H_2S) (Keitel and Häussinger 2018; Fiorucci et al. 2017; Renga et al. 2015a, b). Furthermore, TGR5 also induced gene expression of both eNOS and CSE further supporting generation of vasodilatory molecules (Keitel and Häussinger 2018; Keitel et al. 2007; Fiorucci et al. 2017; Renga et al. 2015a). In contrast, stimulation of TGR5 suppressed expression of endothelin 1 (ET-1), thereby reducing contractility of HSC (Keitel and Häussinger 2018; Fiorucci et al. 2017; Renga et al. 2015b).

Through activation of S1PR2 and TGR5, BAs may exert differential effects in LSECs. While S1PR2 stimulation may promote an inflammatory vascular phenotype, ligand binding to TGR5 triggers vasodilatory effects (Table 1 and Fig. 2b).

6 Role of S1PR2 and TGR5 in Hepatic Stellate Cells (HSCs) and Hepatic Myofibroblasts (hMFs)

S1PR2 has been detected in activated HSCs, myofibroblasts, and fibrotic human liver tissue in colocalization with a-SMA (Li et al. 2011a; Sato et al. 2016). Expression of S1PR2 as well as S1PR1 and S1PR3 was also observed in the human HSC cell line LX-2 (Liu et al. 2011). While siRNA knockdown of S1PR1 and S1PR3 reduced production of extracellular matrix (collagen 1α1, procollagen α1) as well as migratory activity of LX-2 cells in response to S1P, knockdown of S1PR2 enhanced S1P-stimulated migratory activity and had no effect on profibrogenic gene expression (Liu et al. 2011). These data suggest that S1PR1

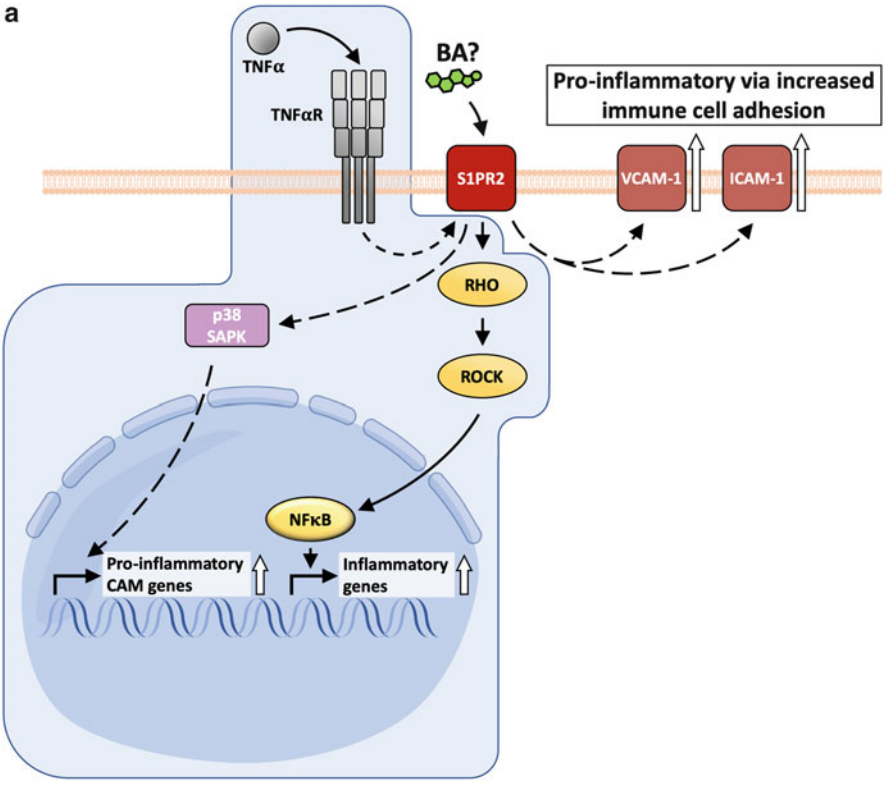

Fig. 2 Role of S1PR2 (**a**) and TGR5 (**b**) in liver sinusoidal endothelial cells. For a detailed description and references refer to text

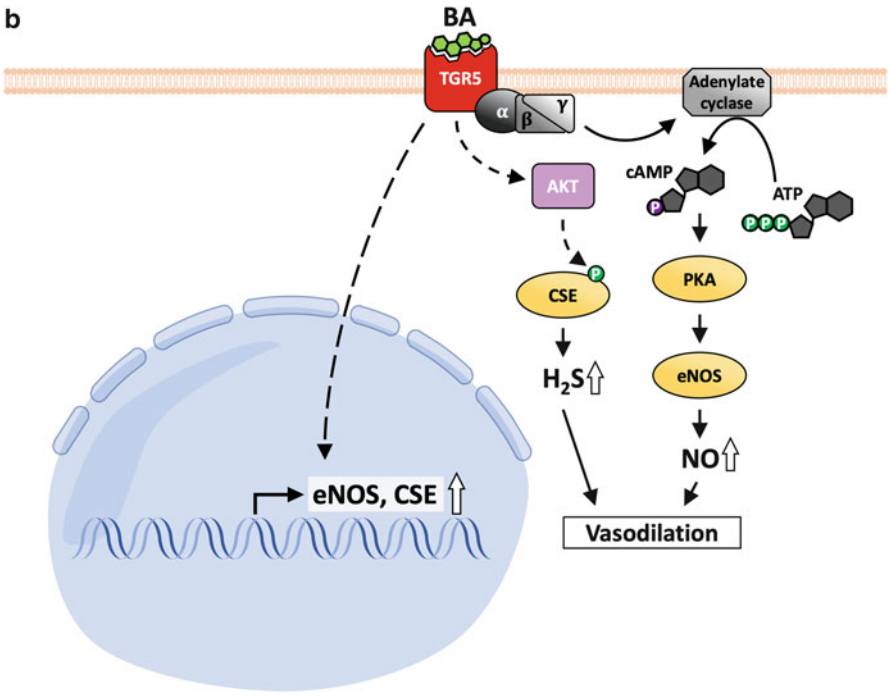

Fig. 2 (continued)

and S1PR3 are important for S1P-induced fibrogenic activation and migration of LX-2 cells, whereas S1PR2 exerts inhibitory effects on migration (Liu et al. 2011). Transfection and stimulation of S1PR2 in LX-2 cells enhanced contractility (Xu et al. 2016).

Expression of TGR5 mRNA and protein are below detection level in quiescent HSCs both in freshly isolated cells in vitro and in liver tissue sections in vivo; however, culturing HSCs on plastic dishes induces TGR5 mRNA levels (Keitel and Häussinger 2018; Keitel et al. 2008b; Sawitza et al. 2015). Thus, TGR5 mRNA and protein levels can be detected in activated myofibroblast-like HSCs in vitro but also in damaged liver tissue in vivo (Keitel and Häussinger 2018; Keitel et al. 2008b; Sawitza et al. 2015). Since cAMP triggers internalization of the endothelin A (ET_A) receptor in activated HSCs, thereby desensitizing the receptor toward ET-1, TGR5 may reduce contractility of HSC by raising intracellular cAMP levels (Keitel and Häussinger 2018; Reinehr et al. 2002). In HSCs, S1PR2 and TGR5 promote opposing effects on contractility.

Table 1 S1PR2 and TGR5 signaling in different liver cell types

Cell type	S1PR2 signaling	TGR5 signaling
Hepatocytes	• ERK1/2, JNK activation • HDAC inhibition • Modulation of gene expression • Inhibits apoptosis	• None
Cholangiocytes	• ERK1/2-mediated cell proliferation, cell migration and invasiveness	• ROS-Src-EGFR-ERK1/2-mediated cell proliferation • cAMP-CFTR-dependent secretion • cAMP-PKA-CD95 anti-apoptotic signaling
HSC	• Rho kinase activation and cell contraction • Cell proliferation • Inhibits migration	• cAMP, ET_A internalization, reduction of contractility
LSEC	• Pro-inflammatory gene expression (ICAM-1, VCAM-1) • JTE-103 inhibits monocyte adhesion	• Upregulation of eNOS and CSE • cAMP-PKA-eNOS-dependent generation of NO • AKT-CSE-dependent generation of H_2S • Reduction in ET-1 expression
Macrophages	• Polarization toward pro-inflammatory M1 phenotype • Secretion of pro-inflammatory cytokines • Enhances migration • Suppresses phagocytosis	• Polarization toward M2, anti-inflammatory phenotype • Reduced secretion of pro-inflammatory cytokines and chemokines, inhibits NLRP3 inflammasome

For a detailed description and references refer to text

7 Role of S1PR2 and TGR5 in Macrophages

Both S1PR2 and TGR5 are highly expressed in macrophages (Kawamata et al. 2003; Hughes et al. 2008). S1PR2 KO mice have a survival advantage over wild-type animals when subjected to the sepsis model of cecal ligation and puncture or to intratracheal *E. coli* inoculation (Hou et al. 2015). This contrasts with findings in TGR5 KO mice, which are more susceptible toward lipopolysaccharide (LPS)-induced sepsis (Wang et al. 2011).

Deletion of S1PR2 in alveolar macrophages or inhibition of S1PR2 in bone marrow-derived macrophages significantly increased phagocytic activity, while bactericidal effects were unaffected (Hou et al. 2015). Interestingly, S1PR2 expression was increased in peripheral blood mononuclear cells (PBMCs) of patients with sepsis, which also showed reduced phagocytic activity in vitro (Hou et al. 2015). While in wild-type macrophages, activation of S1PR2 triggered RhoA-dependent cell contractions and impaired phagocytic activity, absence of S1PR2 promoted Ras GTPase-activating-like protein 1 (IQGAP1)-mediated Rac1 activation resulting in

actin reorganization, lamellipodial protrusion, and enhanced phagocytosis (Hou et al. 2015). Furthermore, S1PR2 promotes differentiation of macrophages into an inflammatory (M1) phenotype. Stimulation of S1PR2 via $G\alpha_i$ resulted in increased PI3K and JNK activation and triggered transcription of pro-inflammatory genes such as TNF-α and monocyte chemoattractant protein-1 (MCP-1, CCL2) (Yang et al. 2018). Furthermore, this S1PR2-$G\alpha_i$-PIK3 signaling pathway also led to activation of the small G protein Rac1, which in turn promoted migration of bone marrow-derived macrophages in vitro or macrophage infiltration into CBDL livers in vivo (Yang et al. 2015).

In contrast, activation of TGR5 promotes polarization of macrophages into an anti-inflammatory phenotype, resulting in reduced expression and secretion of pro-inflammatory cytokines and chemokines (Kawamata et al. 2003; Keitel et al. 2008b; Haselow et al. 2013; Hogenauer et al. 2014; Perino and Schoonjans 2015; Wammers et al. 2018; Guo et al. 2016; Ichikawa et al. 2012; Biagioli et al. 2017). Ligand binding to TGR5 resulted in increased cAMP levels, which inhibited phosphorylation of IκBα and subsequent nuclear translocation of NF-κB p65, thereby preventing NF-κB transcriptional activity (Keitel and Häussinger 2018; Pols et al. 2011b). TGR5 also suppressed caspase-1-mediated maturation of pro-inflammatory cytokines, such as interleukin-1β (IL-1β) and IL-18, through PKA-dependent phosphorylation of nucleotide-binding domain (NB) and leucine-rich repeat (LRR) containing nucleotide oligomerization domain-like receptor protein 3 (NLRP3) (Keitel and Häussinger 2018; Guo et al. 2016). Stimulation of TGR5 through AKT-mTOR induced expression of the CCAAT/enhancer-binding protein β (C/EBPβ) isoform liver inhibitory protein (LIP), which in turn suppressed chemokine transcription (Perino et al. 2014; Perino and Schoonjans 2015). Furthermore, TGR5 impaired macrophage migration and phagocytosis (Kawamata et al. 2003; Perino et al. 2014; Pols et al. 2011b; Perino and Schoonjans 2015).

8 Role of S1PR2 and TGR5 in Biliary Epithelial Cells

S1PR2 mRNA has been detected in murine cell lines derived from small or large bile ducts as well as in various cholangiocarcinoma cell lines (Liu et al. 2015; Wang et al. 2017). Using immunofluorescence staining of mouse, rat, and human tissue, TGR5 has been localized to biliary epithelial cells in the canals of Hering, in small and large intrahepatic bile ducts as well as in extrahepatic bile ducts (Keitel and Häussinger 2011, 2013, 2018; Reich et al. 2016; Keitel et al. 2008b; Soroka et al. 2018). A very high expression of TGR5 is also found in the gallbladder, where the receptor is present in both epithelial cells and in smooth muscle cells (Lavoie et al. 2010; Vassileva et al. 2006; Keitel et al. 2009; Li et al. 2011b). While the subcellular localization of S1PR2 has not been studied in cholangiocytes, TGR5 was detected in the primary cilium, in the apical plasma membrane, in the intracellular vesicular structures, in the endoplasmic reticulum, and in the nuclear membrane (Keitel and Häussinger 2013, 2018; Masyuk et al. 2013; Keitel et al. 2010b, 2015; Deutschmann et al. 2018). The strongest expression of TGR5 was found in the apical, ciliary, and

nuclear membranes (Masyuk et al. 2013; Keitel et al. 2010b). Furthermore, TGR5 was also present on exosomes isolated from rat bile (Masyuk et al. 2013; Deutschmann et al. 2018).

Activation of S1PR2 in large duct cholangiocytes triggered ERK1/2 and AKT activation similar to hepatocytes (Studer et al. 2012; Wang et al. 2017) (Fig. 3a). TC or S1P induced cholangiocyte proliferation in a S1PR2-, ERK1/2-, and AKT-dependent manner (Wang et al. 2017). Expression of S1PR2 mRNA in cholangiocytes was enhanced by TC treatment or by CBDL in vivo (Wang et al. 2017). Furthermore, CBDL-associated cholangiocyte proliferation was abrogated in S1PR2 KO mice (Wang et al. 2017).

Similar to S1PR2, activation of TGR5 by BAs significantly promoted cholangiocyte proliferation both in vitro and in vivo in mice liver following cholic acid (CA) feeding or CBDL (Keitel and Häussinger 2013, 2018; Reich et al. 2016; Deutschmann et al. 2018; Keitel et al. 2015) (Fig. 3b). Thus, targeted deletion of TGR5 in mice resulted in almost complete loss of ductular reaction and cholangiocyte proliferation in response to cholestasis (Reich et al. 2016). The effect of TGR5 on cholangiocyte proliferation, however, is dependent on subcellular localization of the receptor (Keitel and Häussinger 2018; Masyuk et al. 2013; Deutschmann et al. 2018; Keitel et al. 2015). While ligand binding to TGR5 within the primary cilia led to coupling of the receptor to a $G\alpha_i$ protein in H69 cells and thus

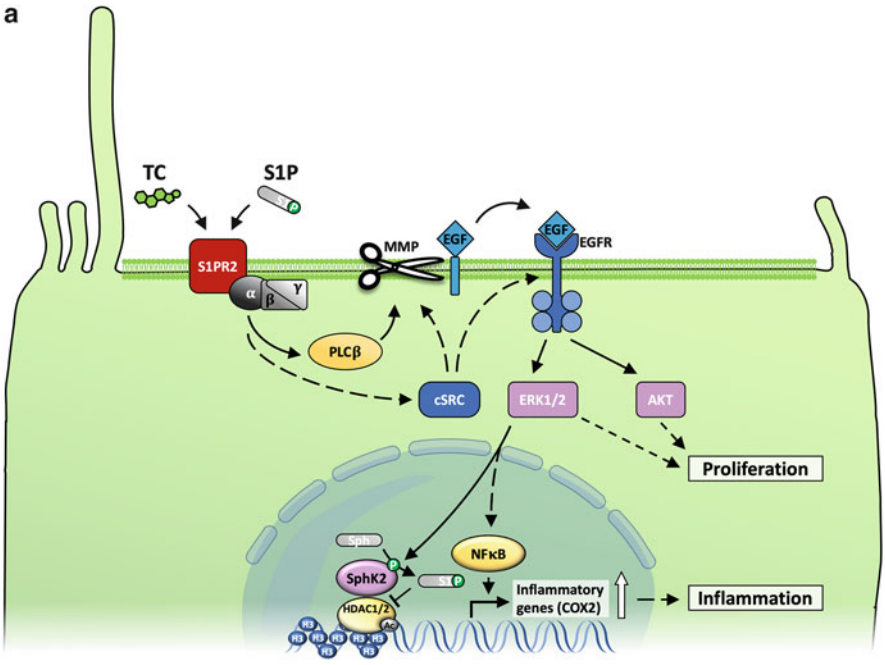

Fig. 3 Role of S1PR2 (**a**) and TGR5 (**b**) in cholangiocytes. For a detailed description and references refer to text

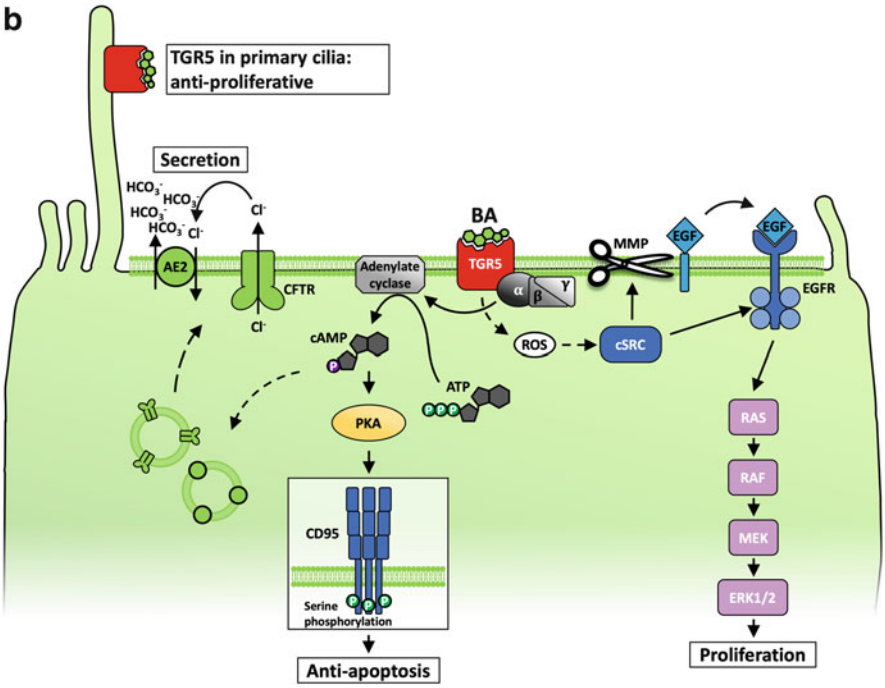

Fig. 3 (continued)

reduction in intracellular cAMP levels and inhibition of cell proliferation, activation of TGR5 in the apical cell membrane of H69 cells triggered proliferation through coupling to $G\alpha_s$ and elevation of cAMP (Masyuk et al. 2013; Reich et al. 2016; Deutschmann et al. 2018). Moreover, cell proliferation could be triggered by TGR5 in primary murine cholangiocytes independently of adenylate cyclase through elevation of ROS and activation of Src kinase, which in turn stimulated matrix metalloproteinases, thereby facilitating EGF shedding and EGFR transactivation leading to phosphorylation of its downstream targets including ERK1/2 (Keitel and Häussinger 2018; Reich et al. 2016; Deutschmann et al. 2018). While cholangiocytes derived from TGR5 KO mice displayed no increased proliferation in response to different BAs or synthetic agonist, cytokine- and growth factor-mediated cell proliferation was comparable to cells derived from wild-type mice (Reich et al. 2016).

Besides proliferation, TGR5 is also essential for cholangiocyte secretion and thus biliary choleresis (Keitel and Häussinger 2018; Li et al. 2011b; Deutschmann et al. 2018; Keitel et al. 2015) (Fig. 3b). Stimulation of TGR5 through $G\alpha_s$ and activation of adenylate cyclase led to a rise in intracellular cAMP and promoted chloride secretion via the cystic fibrosis transmembrane conductance regulator (CFTR, ABCC7) (Keitel et al. 2009). Subsequently, chloride is exchanged against

bicarbonate across the apical membrane by the anionic exchanger 2 (AE2, SLC4A2) promoting bicarbonate-rich choleresis and formation of a bicarbonate layer (known as bicarbonate umbrella) (Keitel and Häussinger 2018; Keitel et al. 2009, 2015; Li et al. 2011b; Deutschmann et al. 2018; Duan et al. 2012; Beuers et al. 2010; Hohenester et al. 2012). Not only transport activity by CFTR but also apical plasma membrane localization of CFTR, AE2, and aquaporin 1 are regulated by cAMP. Elevation of cAMP as mediated through TGR5 triggers insertion of vesicles containing the functionally related proteins CFTR, AE2, and aquaporin 1 from an intracellular vesicular compartment into the apical membrane of cholangiocytes, thus enhancing overall secretory capacity (Keitel and Häussinger 2013; Keitel et al. 2009, 2015; Deutschmann et al. 2018; Cheng et al. 1991; Howard et al. 2000; Tietz et al. 2003). In line with these findings, portal infusion or intraperitoneal injection of a synthetic TGR5 agonist enhanced bile flow significantly in rats and mice, while TGR5 KO mice show reduced bile flow rates (Li et al. 2011b; Keitel et al. 2015).

Cholangiocytes derived from TGR5 KO mice were less resistant toward BA toxicity as well as to death receptor ligand (FAS ligand)-induced apoptosis as compared to cells derived from wild-type animals (Masyuk et al. 2013; Reich et al. 2016; Deutschmann et al. 2018; Keitel et al. 2015). A reduction in protective bicarbonate secretion as well as altered death receptor signaling may account for this susceptibility of TGR5-deficient cells (Keitel and Häussinger 2018; Reich et al. 2016; Li et al. 2011b; Deutschmann et al. 2018; Keitel et al. 2015).

TGR5 is also expressed in the epithelium and the smooth muscle of murine and human gallbladder. While stimulation of TGR5 in the epithelium facilitated secretion, activation of the receptor in smooth muscle cells led to relaxation and thus filling of the gallbladder (Lavoie et al. 2010; Keitel et al. 2009; Li et al. 2011b; Deutschmann et al. 2018). In line with this finding, TGR5 KO mice have a smaller gallbladder volume as compared to their wild-type littermates (Lavoie et al. 2010; Li et al. 2011b). In contrast, application of endogenous or synthetic TGR5 ligands increased gallbladder volume significantly and over twofold (Reich et al. 2017; Li et al. 2011b; Duan et al. 2012; Briere et al. 2015). In contrast, intestine-specific TGR5 agonists with low systemic availability failed to change gallbladder volume significantly (Duan et al. 2015; Chen et al. 2018b). These effects of systemically available TGR5 ligands on gallbladder volume may pose a risk for unwanted side effects if TGR5 agonists proceed to clinical applications (Keitel and Häussinger 2018; Li et al. 2011b).

9 Loss of S1PR2 and TGR5 in Mice and Humans: Contribution to Liver Disease?

Both S1PR2 and TGR5 KO mice are viable and display no overt liver disease phenotype under normal feeding and housing conditions (Adada et al. 2013; Maruyama et al. 2006; Vassileva et al. 2006; Lorenz et al. 2007; MacLennan et al. 2001). However, subtle changes in BA homeostasis have been reported in TGR5 KO mice, in which reduced Cyp7b1 expression and suppression of BA synthesis via the

alternative pathway may explain a smaller BA pool, lower levels of TβMCA, and the relative increase of TC and TDC (Reich et al. 2017; Li et al. 2011b; Donepudi et al. 2017; Häussinger and Keitel 2017). When fed a lithogenic diet, TGR5-deficient mice are resistant to cholesterol gallstone formation, which was explained by increased expression of Cyp7a1 and a lower cholesterol saturation index in bile (Vassileva et al. 2006; Li et al. 2011b). This finding was surprising since gallbladder filling and motility are also impaired in the absence of TGR5, and gallbladder hypomotility should render the animals more susceptible toward gallstone formation (Li et al. 2011b; Deutschmann et al. 2018).

S1PR2 KO mice may suffer from seizures and develop early deafness, and S1PR2 variants have been identified in patients with hearing loss recently (MacLennan et al. 2001; Kono et al. 2007; Santos-Cortez et al. 2016). Variants within the coding sequence of the TGR5 gene were detected in a very low frequency in patients with primary sclerosing cholangitis (PSC) but also in healthy controls (Hov et al. 2010, 2011). Besides these rare non-synonymous variants, a common polymorphism rs11554825 was identified in the noncoding region of exon 1 and is in almost complete linkage disequilibrium with another polymorphism rs3731859 located in the 5′ untranslated region (UTR), which may affect TGR5 mRNA expression levels (Keitel and Häussinger 2018; Deutschmann et al. 2018; Keitel et al. 2015; Hov et al. 2010). The risk allele (C-allele) of rs11554825 was significantly more frequent in patients with PSC across different cohorts as compared to controls (Hov et al. 2010). This rs11554825 variant may act as disease modifier promoting disease progression, since loss of TGR5 renders cholangiocytes more susceptible toward BA toxicity and also promotes a pro-inflammatory phenotype in macrophages both factors contributing to PSC pathogenesis (Keitel and Häussinger 2018; Deutschmann et al. 2018; Keitel et al. 2015; Hov et al. 2010; Hirschfield et al. 2013).

10 S1PR2 and TGR5 in Cholestatic and Biliary Diseases: Pathogenetic Role and Potential Therapeutic Target?

When challenged with CBDL for 14 days, S1PR2 KO mice were partially protected from cholestatic liver injury. S1PR2 KO mice had lower serum and liver BA concentrations, reduced serum levels of alkaline phosphatase (ALP), and showed less hepatic fibrosis and inflammation (Wang et al. 2017). Interestingly, AST and ALT serum levels were comparable between genotypes (Wang et al. 2017). While absence of S1PR2 exerts protective mechanisms by reducing inflammation and liver fibrosis under cholestatic conditions, S1PR2 is upregulated in the liver and cholangiocytes after CBDL and promotes liver injury following CBDL in wild-type animals (Wang et al. 2017). Whether the blunted ductular reaction (DR) in S1PR2 KO mice is responsible for the reduction in fibrosis development is unclear but should be further explored since DR coincides with fibrosis in many conditions (Williams et al. 2014). Similar to CBDL, an upregulation of S1PR2 has been observed in Mdr2 (Abcb4) knockout mice, which develop spontaneous sclerosing cholangitis and biliary fibrosis and serve as animal model for PSC (Wang et al.

2017). In these mice, S1PR2 levels correlated with the degree of fibrosis (Wang et al. 2017). Which of the different S1PR2-expressing cell types contributed to the observed effects remains elusive to date and will need to be addressed before S1PR2 could be considered as therapeutic target.

Using bone marrow chimeras, it could be demonstrated that systemic inhibition of S1PR2 prevented migration of enhanced green fluorescent protein (EGFP)-positive BMDMs into the liver following CBDL for 14 days (Yang et al. 2015). Treatment with JTE-013 reduced serum levels of AST and ALT as well as levels of pro-inflammatory cytokines such as IL-6, TNF-α, MCP-1, and interferon-γ but not of IL-10 (Yang et al. 2015). Furthermore, bile infarcts and fibrosis development as measured by Sirius red staining, hydroxyproline content, and expression of collagen-1α1 and α-smooth muscle actin (α-SMA) were abolished in JTE-013-treated animals (Yang et al. 2015).

In contrast to S1PR2 KO mice, TGR5 KO mice are more susceptible toward cholestatic liver injury (Reich et al. 2016). CBDL for 1–7 days resulted in a more pronounced liver injury, elevated serum MCP-1 levels, and increased IL-1β and TNF-α expression (Reich et al. 2016; Pean et al. 2013). CA feeding (0.5–1%, 5–7 days) also triggered a more pronounced liver damage and attenuated the adaptive hepatocyte and cholangiocyte proliferation (Reich et al. 2016; Pean et al. 2013). Hepatocyte proliferation and liver regeneration were also slowed in TGR5 KO mice after partial hepatectomy as compared to controls (Keitel and Häussinger 2018; Pean et al. 2013).

Analysis of Mdr2 (Abcb4) KO mice revealed reduced levels of TGR5 mRNA and protein in cholangiocytes, which contrasts the upregulation of S1PR2 in these animals (Keitel and Häussinger 2018; Reich et al. 2017; Wang et al. 2017; Deutschmann et al. 2018; Keitel et al. 2015). Furthermore, reduced immunofluorescence staining intensity of TGR5 was observed in cholangiocytes in liver tissue from PSC patients (Keitel et al. 2008a; Reich et al. 2017; Deutschmann et al. 2018; Keitel et al. 2015; Masyuk et al. 2015). Reduced TGR5 levels may hamper the receptor's cytoprotective effects and thus render cholangiocytes more sensitive toward toxic BA effects, as demonstrated for TGR5-deficient murine cholangiocytes (Table 2) (Keitel et al. 2008a, 2015; Reich et al. 2016; 2017; Deutschmann et al. 2018).

BAs, chronic cholestasis, and cholangitis have been implicated in the pathogenesis of hepatobiliary malignancies (Liu et al. 2014; Welzel et al. 2007; Maroni et al. 2014; Yang et al. 2011). Overexpression of both S1PR2 and TGR5 has been demonstrated in human cholangiocarcinoma (CCA) and different CCA cell lines (Reich et al. 2016; Liu et al. 2014, 2015; Deutschmann et al. 2018; Erice et al. 2018).

Stimulation of S1PR2 in different CCA cell lines from humans as well as rats resulted in increased proliferation, migration, and invasiveness. Stimulation of S1PR2 triggered activation of ERK and AKT both directly and indirectly through transactivation of the EGFR. ERK1/2 and AKT subsequently promoted activation of inhibitor of NF-κB kinase (IKK), phosphorylation and degradation of inhibitor of NF-κB (IκB), and translocation of NF-κB into the nucleus resulting in increased COX-2, prostaglandin E_2, IL-6, and TNF-α expression (Liu et al. 2014, 2015). COX-2, PGE_2, and IL-6 have all been associated with CCA proliferation and/or

Table 2 Effects of S1PR2 and TGR5 signaling in different rodent models of liver disease

Disease model	S1PR2	TGR5
Sepsis	• S1PR2 KO mice protected, reduced mortality • JTE-103 reduces pro-inflammatory cytokine expression	• TGR5 KO more susceptible • TGR5 agonists suppress pro-inflammatory cytokine secretion
Cholestasis	• S1PR2 KO protected from injury in CBDL, reduced DR, reduced inflammation, reduced fibrosis • JTE-103 prevents liver damage, reduces BA levels • S1PR2 is upregulated in the liver and cholangiocytes during cholestasis, level of S1PR2 correlates with fibrosis	• TGR5 KO more liver injury (CBDL, BA feeding), reduced DR, more inflammation, reduced hepatocyte proliferation • TGR5 downregulated in Mdr2, PSC • TGR5/FXR dual agonist reduced liver injury in Mdr2 mice
NASH	• S1PR2 KO develop spontaneous lipid accumulation, exaggerated in HFD • JTE-103 protects	• Stimulation of TGR5 promotes energy expenditure, reduces steatohepatitis, reduces obesity, improves glucose tolerance, reduces vascular damage, reduces adipose tissue inflammation, improves kidney injury
Fibrosis	• Stimulation elevates portal pressure • S1PR2 KO have less fibrosis • JTE-103 reduces portal pressure and fibrosis	• Reduces portal pressure through vasodilatory substances and reduction in ET-1 expression • Reduces fibrosis development
CCA	• Overexpressed in human CCA tissue • Promotes proliferation, migration, invasiveness	• Overexpressed in human CCA tissue • Promotes proliferation, migration, invasiveness

For a detailed description and references refer to text

apoptosis resistance (Isomoto et al. 2007; Park et al. 1999; Wu 2005). Overexpression of TGR5 mRNA was found in intrahepatic but also extrahepatic and perihilar CCA tissue (Keitel and Häussinger 2018; Reich et al. 2016; Deutschmann et al. 2018; Erice et al. 2018). Using immunofluorescence staining and quantification, an over threefold increase of TGR5 staining was observed in iCCA cells as compared to nonmalignant cholangiocytes from the non-tumorous resection margin, while cytokeratin-7 staining was comparable (Reich et al. 2016). Stimulation of TGR5 in different CCA cell lines (EGI-1 and TFK-1) triggered cell proliferation through the TGR5-ROS-Src-MMP-EGFR-ERK1/2 pathway as described for primary murine cholangiocytes (Reich et al. 2016). Deletion of TGR5 in these cells completely abolished the proliferative response toward BAs or TGR5 agonists (Reich et al. 2016; Deutschmann et al. 2018). Cell migration and invasiveness increased significantly in response to TGR5 activation and was attenuated in cells with targeted deletion of TGR5 (Reich et al. 2016; Deutschmann et al. 2018; Erice et al. 2018).

Taken together, these data demonstrate that BAs can induce cell proliferation, migration, and invasiveness in CCA through redundant TGR5- and S1PR2-dependent pathways.

While no information is available on the presence of S1PR2 in cystic cholangiocytes of polycystic liver disease, TGR5 is highly expressed in these diseased cells (Masyuk et al. 2015, 2017). Inhibition of TGR5 with a novel inhibitor (SBI-115; m-tolyl 5-chloro-2-[ethylsulfonyl] pyrimidine-4-carboxylate) as well as targeted deletion of TGR5 prevented cell proliferation and cyst growth (Masyuk et al. 2017).

11 S1PR2 and TGR5 Metabolic Liver Diseases: Potential Therapeutic Target?

Nonalcoholic steatohepatitis (NASH) is characterized by hepatic injury with ballooning, inflammation, and varying degrees of fibrosis and occurs in the setting of overnutrition in association with adipose tissue inflammation and insulin resistance (Farrell et al. 2019). Infiltration of Ly-6C^{high} monocytes as well as differentiation of macrophages toward an inflammatory phenotype are characteristics of progressive steatohepatitis (Tacke 2017).

CA feeding prevented HFD-induced weight gain and could also reverse weight gain when added to an already running HFD model in mice, suggesting that BA signaling can prevent obesity (Watanabe et al. 2006).

S1PR2 KO mice when fed a regular chow diet already showed increased lipid accumulation in hepatocytes, which was dramatically increased by high-fat diet (HFD) and accompanied by marked hepatomegaly (Nagahashi et al. 2015). Intrahepatic triglyceride and cholesterol levels were significantly increased in S1PR2 KO mice on HFD (Nagahashi et al. 2015). Whether stimulation of S1PR2 can prevent steatosis development has not been investigated.

When mice on HFD were treated with a TGR5-specific agonist INT-777 (6α-ethyl-23(S)-methyl-cholic acid, EMCA) in addition to HFD, which was started 14 days prior, weight gain could be attenuated, and steatohepatitis could be reversed (Thomas et al. 2009). This was accompanied by a reduction in hepatic triglyceride and fatty acid concentrations, a normalization of serum AST and ALT levels, and significant reduction in serum triglyceride levels (Thomas et al. 2009). The TGR5-dependent beneficial effects on glucose and lipid homeostasis in this model were attributed to induction of energy expenditure as well as increased secretion of glucagon-like peptide-1 (GLP-1) from intestinal enteroendocrine L-cells (Thomas et al. 2009). Treatment of mice with INT-777 prevented atherosclerosis development by reducing inflammation as well as reducing foam cell formation (Pols et al. 2011b).

Exposure of male TGR5 KO mice and wild-type mice for 10 weeks to high-fat cholesterol-enriched and fructose diet (HFD-F) led to a similar weight gain, a similar extent of NASH on histology, development of insulin resistance, and adipose tissue inflammation (Carino et al. 2018). Treatment of wild-type mice with a TGR5-specific agonist BAR-501 (6β-ethyl-3α, 7β-dihydroxy-5β-cholan-24-ol) prevented

weight gain, improved liver serum test, and reversed histological features of NASH including fibrosis and inflammation (Carino et al. 2018). Treatment with BAR-501 also attenuated vascular changes in the aorta, such as thickening of intima and media and inflammation (Carino et al. 2018). Similar results were obtained with a model of menopause-associated obesity and NASH development (de Oliveira et al. 2016). Mice were ovariectomized or sham operated and subsequently fed a HFD for 5 weeks. During the last 4 weeks on HFD, the animals received the FXR agonist OCA or the TGR5 agonist INT-777 (de Oliveira et al. 2016). Treatment with both the FXR agonist and the TGR5 agonist prevents NASH development by reducing accumulation of triglycerides and cholesterol in liver.

Treatment of obese db/db mice with a dual FXR/TGR5 agonist INT-767 (6α-ethyl-24-nor-5β-cholane-3α, 7α, 23-triol-23-sulfate sodium salt) for 6 weeks improved features of steatohepatitis by reducing hepatic steatosis, hepatocyte ballooning, inflammatory infiltrates, and decreased expression of α-SMA and procollagen 1α (McMahan et al. 2013). These findings were accompanied by a decrease in Ly-6C^{high} and increase in Ly-6C^{low} monocyte populations in the liver (McMahan et al. 2013). Stimulation of monocytes with INT-767 resulted in a decrease of Ly-6C expression and polarization into an alternatively activated macrophage (M2) phenotype (McMahan et al. 2013).

The potential role of INT-767 for NASH treatment was further explored in leptin-deficient obese mice fed a high-caloric diet supplemented with cholesterol and trans-fatty acids (AMLN diet) to trigger NASH with varying degrees of fibrosis (Roth et al. 2018). Animals were biopsied before and after 8 weeks of INT-767 treatment (Roth et al. 2018). Oral administration of INT-767 (10 mg/kg) significantly reduced steatosis, hepatocyte ballooning and inflammation, and thus NAS score as well as fibrosis stage (Roth et al. 2018). INT-767 application was compared to treatment with the FXR agonist obeticholic acid (OCA, INT-747, 30 mg/kg), which was applied at a higher dose to account for the fact that INT-767 is about threefold more potent on FXR than OCA (Roth et al. 2018). In comparison, OCA treatment led to similar reduction in NAS score albeit a less efficient lowering of fibrosis stage (Roth et al. 2018). However, the contribution of TGR5 signaling to the reported outcome improvement remains elusive (Roth et al. 2018).

Addition of a novel intestinal TGR5 agonist (RDX8940, EC_{50} $TGR5_{mouse,}$ 0.25 nM; EC_{50} $TGR5_{human}$, 2.5 nM) for 4 weeks to wild-type mice already fed for 10 weeks with Western diet to induce NASH resulted in a significant reduction of liver weight as well as hepatic triglyceride and cholesterol levels (Finn et al. 2019). Application of RDX8940 was associated with increased GLP-1, GLP-2, and PYY secretion (Finn et al. 2019). Likewise, treatment of mice, which were fed with a high-fat, high-carbohydrate diet for 14 weeks for an additional 4 weeks with the GLP-1 analogue liraglutide or the GLP-2 analogue teduglutide, led to a significant drop in serum AST, ALT, and ALP levels, of liver weight as well as of hepatic triglyceride and cholesterol content (Finn et al. 2019). Furthermore, GLP-1 and GLP-2 analogues, respectively, inhibited hepatic expression of profibrogenic genes such as collagen 1α1, tissue inhibitor of metalloproteinases (TIMP), platelet-derived growth factor (PDGF), and transforming growth factor-β (TGF-β) (Finn et al. 2019).

Since alcoholic liver disease shares many features with NASH, it is not surprising that targeting TGR5, FXR, or both in mouse models of acute 3-day binge ethanol exposure or a 12-day prolonged ethanol exposure improved hepatic steatosis and liver inflammation (Iracheta-Vellve et al. 2018). While the FXR agonist OCA (INT-747) was most effective in reducing liver damage in acute 3-day binge ethanol exposure, the dual FXR/TGR5 agonist INT-767 was most effective for preventing liver damage in the prolonged 12-day ethanol exposure model (Iracheta-Vellve et al. 2018).

Therefore, the beneficial effect of TGR5 activation on steatohepatitis may be attributed not only to the systemic anti-inflammatory effects of the receptor on monocytes and macrophages but also on an intact gut-liver axis involving the intestinal secretion of GLP-1 and GLP-2 and other mediators (Pols et al. 2011a; Keitel and Häussinger 2018; Perino and Schoonjans 2015; Carino et al. 2018; Chavez-Talavera et al. 2017).

12 Role in Advanced Liver Disease: Potential Therapeutic Target?

Phenotypic and functional changes occur in LSECs and HSCs during liver fibrogenesis and portal hypertension (PH) development (Iwakiri and Groszmann 2007; Iwakiri et al. 2014; Keitel and Häussinger 2018). LSEC dysfunction in PH is characterized by loss of fenestration, increased expression and release of profibrogenic molecules such as TGF-β and PDGF, deposition of extracellular matrix into the space of Dissé, and impaired generation of vasodilatory mediators such as NO or H_2S (Iwakiri et al. 2014; Keitel and Häussinger 2018). Activation of HSCs triggers transformation into myofibroblast-like cells with loss vitamin A granules, increase production of extracellular matrix, and an upregulation of TGR5 and S1PR2 expression (Sawitza et al. 2015; Iwakiri et al. 2014; Keitel and Häussinger 2018; Kageyama et al. 2012). S1PR2 has also been detected in activated HSCs, myofibroblasts, and fibrotic human liver tissue in colocalization with a-SMA (Li et al. 2011a; Sato et al. 2016). In isolated perfused rat livers, S1P increased portal perfusion pressure, which was inhibited in the presence of the S1PR2 antagonist JTE-103 (Ikeda et al. 2004). Similarly, portal pressure in cirrhotic rats or mice as a result of CBDL for 4 or 3 weeks, respectively, was significantly reduced by infusion of JTE-103 without changes in mean arterial pressure (Kageyama et al. 2012). This reduction in portal pressure was not observed in control animals (Kageyama et al. 2012). Inhibition of S1PR2 was associated with reduction in Rho kinase activity in cirrhotic animals (Kageyama et al. 2012). S1PR2 expression increased in CBDL cirrhotic livers and S1PR2 was predominately found in α-SMA-positive cells (Kageyama et al. 2012).

Inhibition of S1PR2 signaling in active stellate cells prevented contraction as well as Rho activation (Ikeda et al. 2004). Similar results have been shown in a HSC cell line (LX-2), where transfection and stimulation of S1PR2 resulted in strong contraction (Xu et al. 2016).

Treatment with carbon tetrachloride (CCl_4) for 4 weeks triggered extensive fibrosis development in wild-type mice but not in S1PR2 KO mice despite similar liver damage as measured by comparable elevation of serum ALT between both genotypes (Ikeda et al. 2009). The livers of S1PR2 KO mice showed less accumulation of a-SMA-positive cells (Ikeda et al. 2009).

In human fibrotic liver samples, conflicting data exist regarding S1PR2 expression. While a significant suppression of S1PR2 expression and a significant reduction in S1PR2 protein levels were reported in one study (Li et al. 2011a), an upregulation of S1PR2 expression was found in another study of fibrotic liver tissue (Sato et al. 2016). The latter finding is consistent with the animal studies described above (Kageyama et al. 2012).

Modulation of portal pressure and liver microcirculation have been attributed to TGR5 activation already under physiological conditions (Keitel et al. 2007; Keitel and Häussinger 2018). In CCl_4-treated mice with advanced liver fibrosis, stimulation of TGR5 with BAR501 reduced portal hypertension significantly (Reich et al. 2017; Renga et al. 2015b). The molecular mechanisms were increased expression and activity of CSE, resulting in generation of H_2S and simultaneous reduction of ET-1 expression (Renga et al. 2015b; Keitel and Häussinger 2018). Moreover, internalization of the ET_A receptor via cAMP reduced responsiveness of activated HSC toward ET-1 and may also alleviate portal hypertension (Reinehr et al. 2002). Albeit the reduction in portal pressure, expression levels of profibrogenic genes, such as TGF-β, collagen 1α1, and α-SMA, were unaltered (Renga et al. 2015b). However, as described above in NASH models, stimulation of TGR5 may also reduce liver fibrosis (Carino et al. 2018; Roth et al. 2018).

13 Summary and Perspectives

BA effects in the liver are mediated through different BA receptors, including the GPCRs S1PR2 and TGR5. S1PR2 is localized in all liver cell types, including hepatocytes. Activation of S1PR2 promotes pro-inflammatory, profibrogenic, and proliferative effects. Thus, stimulation of S1PR2 may accelerate disease progression. Overexpression of S1PR2 has been detected in fibrotic human livers as well as in cholangiocarcinoma tissue. Inhibition of S1PR2 signaling or targeted deletion of S1PR2 prevented liver damage induced by sepsis, cholestasis, high-fat diet, or carbon tetrachloride treatment.

Similar to S1PR2, TGR5 (GPBAR1) is expressed in different non-parenchymal liver cells, where the receptor modulates liver microcirculation, inflammatory response, and biliary function. Mice with targeted deletion of TGR5 are more susceptible toward liver damage in response to BA feeding, partial hepatectomy, common bile duct ligation, or treatment with lipopolysaccharide. Stimulation of TGR5 improves many aspects of the metabolic syndrome, including steatohepatitis, glucose homeostasis, adipose tissue inflammation, atherosclerosis, kidney injury, as well as obesity through increased energy expenditure, enhanced lipolysis, and mitochondrial biogenesis. However, TGR5 activation triggers gallbladder filling

via gallbladder smooth muscle relaxation, which may limit the use of systemically available highly potent TGR5 agonists. Intestine-specific TGR5 ligands with low systemic availability promote GLP-1 and GLP-2 secretion, ameliorate insulin resistance and improve steatohepatitis, and are devoid of the unwanted side effects on gallbladder function; however, some of the beneficial effects of targeting systemic TGR5 especially with regard to the anti-inflammatory functions will not be mimicked by these substances.

Similar to S1PR2, an overexpression of TGR5 was detected in cholangiocarcinoma tissue and CCA cell lines, where activation of the receptor resulted in increased cell proliferation, migration, and invasiveness. Furthermore, high TGR5 levels are found in cystic cholangiocytes and inhibition of TGR5 signaling prevented cyst growth and cell proliferation in polycystic liver disease. Thus, inhibition of TGR5 may prove useful in some diseases.

Further studies will be needed to decipher the role of different BA receptors in the liver and also to study the interplay of these receptors under physiological and disease conditions. Due to the broad expression profile and the pleiotropic functions of S1PR2 and TGR5, targeting these receptors without creating a plethora of unwanted effects will be challenging. Selective receptor modulators, which have tissue-specific targeting and may even be able to dissociate metabolic, inflammatory, and proliferative signaling, would overcome these problems.

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Bile Acid-Activated Receptors: A Review on FXR and Other Nuclear Receptors

Dong-Ju Shin and Li Wang

Contents

D.-J. Shin (✉)
Department of Physiology and Neurobiology, University of Connecticut, Storrs, CT, USA
e-mail: dong-ju.shin@uconn.edu

L. Wang
Department of Physiology and Neurobiology, University of Connecticut, Storrs, CT, USA

Veterans Affairs Connecticut Healthcare System, West Haven, CT, USA
e-mail: li.wang@uconn.edu

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Abstract

Nuclear receptors (NRs) are ligand-dependent transcription factors that are involved in various biological processes including metabolism, reproduction, and development. Upon activation by their ligands, NRs bind to their specific DNA elements, exerting their biological functions by regulating their target gene expression. Bile acids are detergent-like molecules that are synthesized in the liver. They not only function as a facilitator for the digestion of lipids and fat-soluble vitamins but also serve as signaling molecules for several nuclear receptors to regulate diverse biological processes including lipid, glucose, and energy metabolism, detoxification and drug metabolism, liver regeneration, and cancer. The nuclear receptors including farnesoid X receptor (FXR), pregnane X receptor (PXR), constitutive androstane receptor (CAR), vitamin D receptor (VDR), and small heterodimer partner (SHP) constitute an integral part of the bile acid signaling. This chapter reviews the role of the NRs in bile acid homeostasis, highlighting the regulatory functions of the NRs in lipid and glucose metabolism in addition to bile acid metabolism.

Keywords

Bile acids · Metabolism · Nuclear receptor

1 Introduction

Nuclear receptors (NRs) are a family of transcription factors that regulate their target genes in response to their ligands, carrying out their functions in various biological processes including metabolism, reproduction, and development. Since the cloning of the first member of the family in 1985, many members of the NR family have been discovered, comprising 48 members in the human genome today (Chawla et al. 2001). The family includes classic endocrine receptors that are activated by steroid hormones, thyroid hormones, and retinoids, as well as orphan NRs whose ligands have not been identified yet. Although the NR family members exhibit a wide variations in their ligand sensitivity, they generally share a common structural organization that consists of a ligand-independent transcriptional activation function (AF-1) domain (A/B), a core DNA-binding domain (C), a hinge region (D), a COOH-terminal ligand-binding domain (E), and a ligand-dependent activation function (AF-2) domain (F) (Fig. 1a). Ligand binding to a NR leads to a conformational change in the receptor, facilitating the replacement of a corepressor with a coactivator, enabling the transcription to occur to induce their target gene expression (McKenna et al. 1999). The classic steroid hormone receptors bind to their specific DNA response elements as a homodimer. On the contrary, adopted orphan receptors and orphan receptors whose endogenous ligands remain unidentified bind to their DNA response elements as a heterodimer with the retinoid X receptor (RXR), while some NRs bind to their DNA as a monomer (Fig. 1b, c). The architectures of the DNA response element are diverse from a palindrome and direct repeat to a

a

b

Group	Nuclear receptor
Endocrine receptors	GR, ERα, ERβ, PR, AR, MR, RARα, RARβ, RARγ, TRα, TRβ, VDR, EcR
Adopted orphan receptors	RXRα, RXRβ, RXRγ, PPARα, PPARβ, PPARγ, LXRα, LXRβ, FXR, PXR, CAR
Orphan receptors	SF-1, LRH1, DAX-1, PNR, GCNF1, SHP, NGFI-Bα, NGFI-Bβ, NGFI-Bγ, ERRα, ERRβ, ERRγ, RORα, RORβ, RORγ, HNF4, COUP-TFα, COUP-TFβ, COUP-TFγ, TR2, TR4, REV-ERBα, REV-ERBβ, REV-ERBγ

c

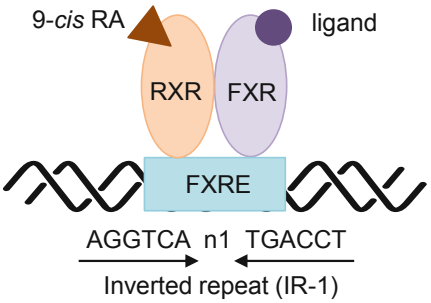

Fig. 1 (**a**) Schematic view of nuclear receptor structure, (**b**) subgroups of nuclear receptor family members based on the source and type of their ligands, (**c**) the binding of FXR/RXR heterodimer to FXRE (IR-1) is shown as an example (adopted from Chawla et al. 2001)

monomeric site of a consensus half-site 5′AGGTCA3′ with a various number of nucleotides in the space (Chawla et al. 2001; Khorasanizadeh and Rastinejad 2001).

Bile acids are synthesized from cholesterol exclusively in the liver via two different pathways, the classic (neutral) pathway and the alternative (acidic) pathway (Fig. 2). The classic pathway which accounts for more than 90% of total bile acid production in human is initiated by microsomal cholesterol 7alpha-hydroxylase (CYP7A1), the rate limiting enzyme, producing cholic acid (CA) and chenodeoxycholic acid acid (CDCA), whereas the alternative pathway is initiated by sterol-27-hydroxylase (CYP27A1), predominantly generating CDCA. The primary bile acids, CA and CDCA, generated by the liver are conjugated with glycine and to a less extent with taurine in humans and secreted to the gall bladder where they are stored. Upon food ingestion, the gall bladder undergoes contractions, which

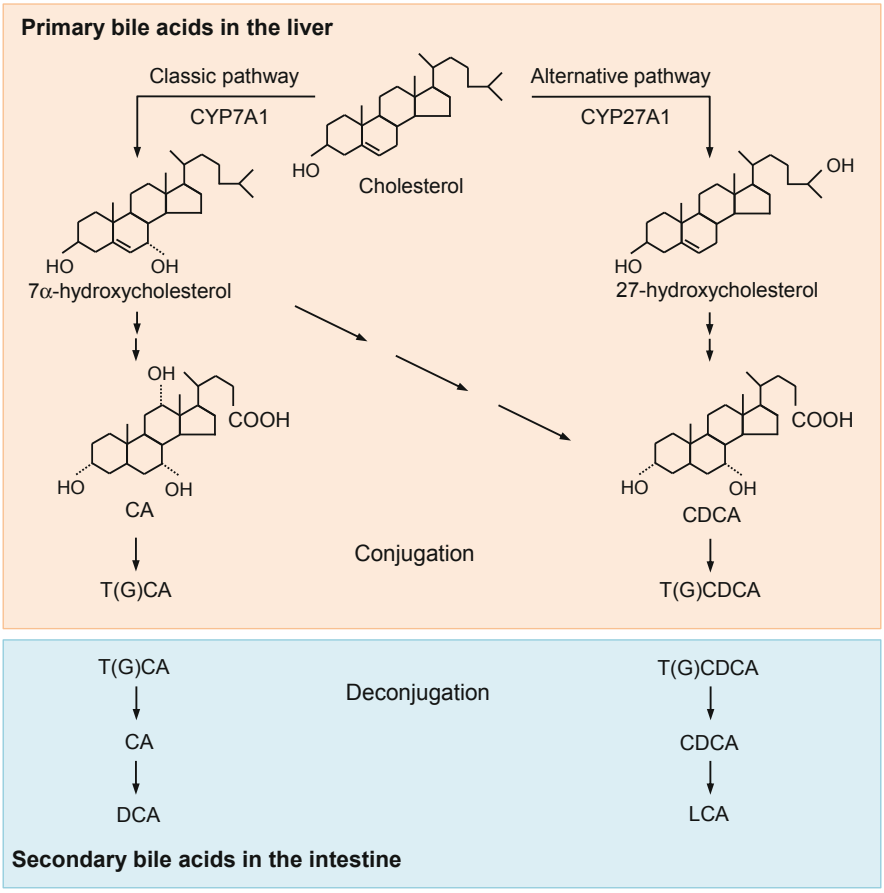

Fig. 2 Schematic view of biosynthetic pathways of primary bile acids in the liver and secondary bile acids in the intestine. *G* glycine-conjugated species, *T* taurine-conjugated species (adopted from Wahlstrom et al. 2016)

in turn promotes the release of bile acids to the intestine where they facilitate the digestion and absorption of lipids and fat-soluble vitamins. Ninety-five percent of bile acids are reabsorbed in the ileum and returned to the liver through the portal vein, a process called enterohepatic circulation. The remaining bile acids are excreted to the feces, which serves as a major route to remove cholesterol from the body. In the intestine, some bile acids are deconjugated and converted to deoxycholic acid (DCA) and lithocholic acid (LCA), secondary bile acids, by gut bacteria with bile salt hydrolases (Li and Chiang 2014; Wahlstrom et al. 2016).

Studies over the past two decades have shown that bile acids are not only considered as emulsifiers for the digestion of lipids and fat-soluble vitamins but also serve as signaling molecules to activate NRs and G protein-coupled bile acid receptor 1 (GPBAR1, also known as TGR5) (Lefebvre et al. 2009). Among the NRs,

farnesoid X receptor (FXR), pregnane X receptor (PXR), and vitamin D receptor (VDR) are directly bound to bile acids and are regarded as bile acid sensors. Although small heterodimer partner (SHP) and constitutive androstane receptor (CAR) have not been shown to be directly bound to bile acids as their ligands, they are important components in bile acid signaling. As bile acids play a critical role in regulating lipid, glucose, and energy metabolism in addition to regulating their own synthesis, in this chapter we will discuss the regulatory roles of FXR, PXR, CAR, VDR, and SHP in the metabolic pathways.

2 Bile Acid-Activated Receptor Farnesoid X Receptor (FXR, NR1H4)

The FXR was initially identified as an orphan NR activated by farnesol derivatives, key intermediates of the mevalonate pathway (Forman et al. 1995). Subsequent studies have identified that bile acids such as CDCA being the most potent in the order of CDCA > LCA = DCA > CA function as endogenous ligands for FXR (Table 1). It has been now well established that FXR is the primary bile acid sensor (Makishima et al. 1999; Parks et al. 1999). FXR is expressed predominantly in the liver and intestine which are major organs that play key roles in maintaining bile acid homeostasis (Forman et al. 1995; Seol et al. 1995). It is also expressed in other tissues at lower levels, including kidney, white adipose tissue, and adrenal gland, although the biological functions of FXR have not been well established in those tissues.

There are two FXR genes, FXR (also known as NR1H4) and FXRβ (also known as NR1H5). The FXR gene produces four functional transcript variants, FXRα1, FXRα2, FXRα3, and FXRα4, that are generated by alternative promoter usage and alternative splicing (Huber et al. 2002; Zhang et al. 2003). The FXR isoforms are biologically functional in response to bile acids, whereas FXRβ is responsive to lanosterol in mice, rats, rabbits, and dogs but constitutes a pseudogene in humans and primates (Otte et al. 2003).

Upon activated by its ligands, FXR interacts with its heterodimer partner RXR and binds to its specific DNA response element, an FXR response element (FXRE), mainly composed of an inverted repeat of two half-sites separated by one nucleotide

Table 1 NRs and their endogenous bile acid ligands

	Endogenous ligands	Tissues	References
FXR	CDCA > LCA=DCA>CA	Liver, Intestine	Makishima et al. (1999) Parks et al. (1999)
PXR	3-keto-LCA > LCA	Liver, intestine	Staudinger et al. (2001)
VDR	LCA > 3-keto-LCA > glyco-LCA > 6-keto-LCA LCA acetate LCA propionate	Intestine	Makishima et al. (2002) Adachi et al. (2005) Ishizawa et al. (2008)

(IR-1) (Pircher et al. 2003; Song et al. 2001), thereby regulating transcription of its target genes. Additionally, the transcriptional activity of FXR is modulated by posttranslational modifications such as phosphorylation (Gineste et al. 2008), acetylation (Kemper et al. 2009), sumoylation (Balasubramaniyan et al. 2013), and O-GlcNAcylation (Berrabah et al. 2014) in response to a synthetic ligand for FXR.

2.1 FXR and Bile Acid Metabolism

Consistent with its high expression in the liver and intestine, FXR regulates transcription of genes involved in bile acid synthesis, absorption, uptake, and transport in the tissues, maintaining bile acid homeostasis. FXR deficiency in mice results in disturbance of bile acid homeostasis accompanied with disordered metabolic homeostasis, demonstrating the crucial role of FXR in bile acid homeostasis and metabolic regulation (Ma et al. 2006; Sinal et al. 2000).

In liver, FXR inhibits bile acid synthesis through a feedback mechanism that involves SHP. Upon activation by its ligand, FXR induces transcription of SHP, an atypical NR acting as a corepressor, which in turn directly interacts with liver-related homolog-1 (LRH-1), a competent transcription factor for Cyp7a1, and inhibits the transcriptional activity of LRH-1, repressing Cyp7a1, the rate limiting enzyme in bile acid synthesis (Goodwin et al. 2000; Lu et al. 2000). Similarly, SHP interacts with hepatocyte nuclear factor (HNF) 4α to inhibit its transcriptional activity, leading to the repression of expression of sterol 12-alpha-hydroxylase (CYP8B1) that is involved in bile acid synthesis (Zhang and Chiang 2001). Additionally, FXR induces fibroblast growth factor 15 (FGF15; FGF19 in humans) in the intestine in response to bile acids, which travels through the portal vein to the liver where it inhibits Cyp7a1 transcription via its receptor, FGF receptor 4 (FGFR4)-mediated signaling pathway (Inagaki et al. 2005). In hepatocytes, FXR controls intracellular bile acid concentrations by regulating genes in bile acid uptake and export. FXR downregulates sodium taurocholate cotransporting polypeptide (NTCP, also known as SLC10A1) (Denson et al. 2001) and organic-anion-transporting polypeptide (OATP, also known as SLCO1A2), which uptake bile acids that are reabsorbed from the intestine and reach to the liver through the portal vein. On the other hand, FXR upregulates apical bile acid efflux transporters, bile salt export pump (BSEP, also known as ABCB11) (Ananthanarayanan et al. 2001), and multidrug resistance-associated protein 2 (MRP2, also known as ABCC2) (Kast et al. 2002), which promote the secretion of bile acids into the gall bladder. Multidrug resistance protein 2 (MDR2) and MDR3 (also known as ABCB4) (Liu et al. 2003), phosphatidylcholine transporters, are also upregulated by FXR. At the basolateral membrane, FXR induces expression of organic solute transporter alpha (OSTα) and OSTβ to efflux bile acids to the circulation (Boyer et al. 2006). Overall, FXR prevents the accumulation of bile acids by the coordinated regulation of gene expression, protecting the liver from the toxic effects of bile acids, which occurs when they are highly accumulated.

In the intestine, FXR inhibits expression of apical sodium-dependent bile salt transporter (ASBT, also known as SLC10A2 or ileal bile acid transporter (IBAT)) on the apical membrane of enterocytes in mice and humans (Chen et al. 2003; Neimark et al. 2004), reducing the absorption of bile acids to enterocytes. Conversely, FXR upregulates ileal bile acid-binding protein (IBABP, also known as gastrotropin) (Grober et al. 1999) that promotes intracellular movement of bile acids from the apical to the basolateral membrane where FXR activated by the increased bile acids induces expression of OSTα and OSTβ (Landrier et al. 2006), which leads to the release of bile acids to the portal venous system that delivers bile acids to the liver. As a consequence, FXR prevents intestinal cellular injury that can arise from high toxic concentrations of bile acids similarly in liver.

2.2 FXR and Lipid Metabolism

Earlier studies have shown that CDCA treatment is efficacious to alleviate serum hypertriglyceridemia in men, demonstrating the lipid-lowering potential of bile acids (Bateson et al. 1978; Leiss and von Bergmann 1982). In line with the observations, studies have shown that FXR in response to bile acids plays a significant role in maintaining lipid homeostasis. FXR deficiency is associated with markedly elevated serum and hepatic triglyceride and cholesterol levels (Lambert et al. 2003; Sinal et al. 2000), whereas treatment of GW 4064, a selective agonist for FXR, lowers hyperlipidemia in db/db mice (Zhang et al. 2006). Although the underlying molecular mechanisms behind the FXR's effects on improving lipid profiles remain incompletely understood, it was suggested that FXR regulates multiple steps in lipid homeostasis including hepatic lipogenesis, lipid oxidation, clearance, uptake, and transport. In the liver, the FXR-SHP axis in response to bile acids has shown to inhibit the expression of sterol regulatory binding protein 1c (Srebp1c) along with key lipogenic genes such as stearoyl-CoA desaturase-1 (Scd-1) and acyl-CoA synthetase short chain family member 2 (Acss2) (Watanabe et al. 2004). On the other hand, activation of FXR induces the expression of peroxisome proliferator-activated receptor (Ppar) α and its targets (Pineda Torra et al. 2003) and the expression and secretion of hepatic fibroblast growth factor 21 (FGF21) (Cyphert et al. 2012), both of which are involved in fatty acid oxidation. Additionally, FXR controls genes in lipoprotein metabolism. It has been suggested that expression of ApoE, phospholipid transfer protein (Pltp), and very low density lipoprotein receptor (Vldlr) is directly upregulated by FXR (Mak et al. 2002; Sirvent et al. 2004). Ligand-activated FXR induces expression of apolipoprotein C2 (ApoC2) (Kast et al. 2001), whereas it reduces ApoC3 (Claudel et al. 2003), which leads to reducing plasma triglyceride levels. ApoC2 and ApoC3 are a component of very low density lipoproteins. While ApoC2 activates lipoprotein lipase which hydrolyzes triglycerides into free fatty acids, lowering plasma triglycerides, ApoC3 inhibits lipoprotein lipase enzyme activity, leading to a delayed hydrolysis of triglycerides. Therefore, through the coordinated regulation of ApoC2 and ApoC3, FXR may control lipid clearance and transport, contributing to the improvement of lipid profile.

2.3 FXR and Glucose Metabolism

Studies have shown that bile acids and FXR agonists control glucose homeostasis. In mice, CDCA and FXR agonist GW4064 downregulate expression of hepatic gluconeogenic genes such as phosphoenolpyruvate carboxykinase (Pck1) and glucose-6-phophatase (G6pc), lowering plasma glucose levels concomitantly with improved insulin sensitivity in db/db diabetic mice (Ma et al. 2006; Yamagata et al. 2004; Zhang et al. 2006). Consistently, hepatic overexpression of FXR improves hyperglycemia in diabetic mice, whereas FXR deficiency leads to glucose intolerance and insulin resistance (Zhang et al. 2006). Additionally, a recent study has shown that FXR activation induces expression of aldo-keto reductase 1B7 (Akr1b7) and that hepatic overexpression of Ark1b7 is associated with reduced hepatic gluconeogenesis and plasma glucose levels without improvement of insulin sensitivity (Ge et al. 2011). However, other studies have shown that activation of FXR by its ligands upregulates expression of Pck1 in human and rat hepatocytes (Stayrook et al. 2005), suggesting that the underlying mechanisms of the beneficial effects of FXR on hepatic gluconeogenesis remain incompletely understood. Interestingly, hepatic-specific deletion of FXR mice on a high-fat diet fails to improve glucose homeostasis and insulin resistance (Prawitt et al. 2011), while hepatic-specific double deletion of FXR and SHP mice leads to improved glucose tolerance and insulin sensitivity along with reduced hepatic triglyceride accumulation and adiposity (Kim et al. 2017), suggesting a potential role for non-hepatic FXR or additional factors in controlling glucose homeostasis.

It has been shown that the intestine plays a role in maintaining the whole-body glucose homeostasis. FXR in response to bile acids induces fibroblast growth factor FGF19 (FGF15 in mice), a postprandial enterokine that is synthesized in and released from the intestine to the liver where it increases hepatic glycogen synthesis (Kir et al. 2011). In mice, intestinal FGF15 induced by FXR leads to inactivation of cAMP regulatory element-binding protein (CREB) and downregulation of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), inhibiting hepatic gluconeogenesis. Additionally, FXR knockout mice display delayed intestinal glucose absorption, although the underlying mechanisms of the alterations remain elusive (van Dijk et al. 2009). Interestingly, a recent study demonstrated that the intestine-restricted FXR agonist fexaramine shapes the gut microbiota to increase TGR5-induced glucagon-like peptide-1 (GLP-1) secretion to improve hepatic glucose profile and insulin sensitivity (Pathak et al. 2018).

2.4 FXR as a Therapeutic Target

Consistent with the crucial role of FXR in regulating lipid and glucose metabolism and energy metabolism in addition to bile acid synthesis, activation of FXR by its ligands has been shown to be beneficial to improve various metabolic diseases,

including non-alcoholic fatty liver disease (NAFLD), type 2 diabetes, obesity, and cholestasis (Chavez-Talavera et al. 2017; Lee et al. 2006; Teodoro et al. 2011). Obeticholic acid (OCA, 6α-ethyl-chenodeoxycholic acid (6-ECDCA), also known as INT-747), a semisynthetic variant of chenodeoxycholic acid, displays a potent FXR agonist effect. It exhibits 100-fold greater FXR agonist activity than CDCA in reporter gene assays (Pellicciari et al. 2002). OCA has been recently approved by the US Food and Drug Administration (FDA) to treat primary biliary cholangitis (PBC), a chronic cholestatic liver disease where the bile ducts in the liver are damaged, leading to building up bile acids in the liver causing biliary fibrosis and eventually cirrhosis. The clinical study of OCA for nonalcoholic steatohepatitis (NASH) and alcoholic hepatitis (AH) has been recently completed (NCT01265498 and NCT02039219), and the study of OCA for lipodystrophy and NASH with fibrosis is currently recruiting (NCT02430077 and NCT02548351). OCA has been shown to improve insulin sensitivity and regulate glucose homeostasis. It also reduces markers of liver inflammation and fibrosis in type 2 diabetes and NAFLD (Adorini et al. 2012; Mudaliar et al. 2013) and liver cirrhosis in thioacetamide (TAA)-treated rats (Verbeke et al. 2016) and displays anticholestatic activity as well (Pellicciari et al. 2002). Additionally, a recent study has shown that OCA is effective to treat PBC patients who had an inadequate response to ursodeoxycholic acid (UDCA) (Hirschfield et al. 2015), which is a previously approved drug to treat PBC. In addition to OCA, several other ligands for FXR such as INT-767, Px-102, LJN452, and WAY-362450 are being evaluated for the efficacy for drug development (for details, see Sepe et al. 2018).

3 Bile Acids and Small Heterodimer Partner (SHP, NR0B2)

The SHP was originally cloned in the yeast two-hybrid system during isolating an interaction partner for CAR (Seol et al. 1996). Unlike other NR family members, SHP lacks the conserved DNA-binding domain, functioning as a transcriptional co-regulator. It interacts with a variety of NRs such as RXR, retinoic acid receptors (RARs), HNF4α, LRH-1, estrogen-related receptor (ERR), liver X receptor (LXR), PPARs, glucocorticoid receptor (GR), estrogen receptor (ER), TRβ, FXR, PXR, CAR, androgen receptor (AR), and NGFI-B, (Nur77) and interferes with their transcriptional activity (Seol et al. 1996, 1997; Zhang et al. 2011). Additionally, it interacts with non-NRs including c-Jun, SREBP-1, and brain and muscle ARNT-Like 1 (BMAL1) (Chanda et al. 2008). Although SHP contains a conserved putative ligand-binding domain, its specific natural ligand has not been identified. SHP is predominantly expressed in the liver and gallbladder and at lower levels in the pancreas, brain, stomach, intestine, kidney, ovary, testis, and heart (Bookout et al. 2006).

3.1 SHP and Bile Acid Metabolism

SHP plays a critical role in bile acid homeostasis, even though bile acids have not been shown to serve as ligands for SHP. Rather, bile acids activate FXR that induces

transcription of Shp, which in turn interferes with the critical transcription factors to inhibit the transcription of Cyp7a1 (Goodwin et al. 2000; Lee et al. 1999). The role of the FXR-SHP-LRH-1 axis is well described in a negative feedback inhibition of Cyp7a1 and bile acid synthesis. In addition to the transcriptional regulation of Shp by FXR, the activity of SHP is modulated at the posttranscriptional level, resulting in disturbance of bile acid homeostasis. SHP mRNA stability is reduced by maternally expressed gene 3 (MEG3), a long noncoding RNA, which interacts with a RNA-binding protein polypyrimidine tract-binding protein 1 (PTBP1), guiding it to SHP mRNA to facilitate the direct interaction with SHP mRNA and degradation (Zhang et al. 2017).

Similarly, B-cell lymphoma protein 2 (Bcl2), a founding member of the regulator protein family regulating cell cycle, rapidly induces SHP protein degradation, accompanied with increased expression of long noncoding RNA H19 (Zhang et al. 2016). The reduced levels of SHP in the studies were highly associated with increased hepatic bile acid accumulation and liver injury. Consistently, two studies have demonstrated that SHP knockout mice are defective in maintaining bile acid homeostasis. They exhibit abnormal accumulation and increased synthesis of bile acids due to derepression of Cyp7a1 and Cyp8b1 in response to a specific agonist for FXR (Kerr et al. 2002; Wang et al. 2002). However, the repression of Cyp7a1 and Cyp8b1 in response to bile acids is retained in SHP knockout mice, indicating that SHP-independent compensatory repression pathways are present regulating bile acid homeostasis. As an alternative inhibitory pathway, activation of PXR and the c-Jun N-terminal kinase JNK has been suggested to repress Cyp7a1 and bile acid synthesis (Kerr et al. 2002; Wang et al. 2002).

In addition to the regulatory role of SHP in bile acid synthesis, SHP regulates expression of genes in bile acid transport to maintain intracellular bile acid levels in liver. In cholestasis associated with abnormal retention of bile acids, SHP reduces expression of Ntcp, the major bile acid transporter in the hepatocellular bile acid uptake (Zollner et al. 2002). Additionally, SHP represses organic-anion-transporting polypeptide OATP-C (solute carrier gene family SLC21A6), in response to bile acids by inhibiting transcriptional activity of HNF4α that increases expression of HNF-1α, a critical transcriptional activator for OATP-C (Jung and Kullak-Ublick 2003). It has also been shown that the expression of human organic anion transporter (OAT) 2 (SLC22A7) is reduced by SHP through the inhibition of transcriptional activity of HNF4α, an activator for hOAT2 gene transcription (Popowski et al. 2005). In the intestine, expression of ASBT that promotes uptake of bile acids particularly conjugated bile acids is reduced by SHP in response to bile acids in human and mice, but not in rats, which leads to a reduced influx of bile acids into hepatocytes via the enterohepatic circulation (Chen et al. 2003; Neimark et al. 2004). Overall, the studies suggest that SHP plays a protective role in preventing the accumulation of bile acids in the liver and liver damage from the harmful effects of potentially toxic bile acids.

3.2 SHP and Lipid Metabolism

In line with the general agreement of the beneficial effects of bile acids on lowering serum triglyceride levels, studies have shown that SHP in response to bile acids regulates lipid metabolism. Transgenic mice constitutively over-expressing SHP in the liver display the depletion of hepatic bile acid pool with concomitant accumulation of triglycerides in the liver. It was proposed that the altered phenotypes of the SHP transgenic mice were possibly due to SHP-dependent direct repression of downstream target genes and the bile acid receptor FXR accompanied with an indirect activation of PPARγ and SREBP-1c, and its lipogenic downstream targets including fatty acid synthase (Fasn), acetyl-CoA carboxylase alpha (Acaca), and Scd-1 (Boulias et al. 2005). Additionally, inhibition of SREBP-1c transcription by SHP in response to bile acids was reported in mice followed by reduced expression of acetyl-CoA synthetase (AceCS) and Scd-1, downstream targets of SREBP-1c (Watanabe et al. 2004), suggesting the inhibitory role of SHP in triglyceride accumulation. Consistently, deletion of SHP in obese leptin-deficient ob/ob mice leads to increased hepatic very-low density lipoprotein (VLDL) secretion and elevated microsomal triglyceride transfer protein (MTTP), preventing the development of fatty liver (Huang et al. 2007). Disruptions of hepatic lipid homeostasis can occur, when circadian rhythm undergoes derangement. Transcription of SHP is under the control of circadian activators, circadian locomotor output cycles kaput (CLOCK), and BMAL1 synergistically with LRH-1 (Oiwa et al. 2007). Accordingly, studies have shown that SHP cross-talks with circadian components neuronal PAS domain protein 2 (NPAS2), RAR-related orphan receptor (ROR) α, RORγ, and REV-ERBα (Lee et al. 2015), and modulates circadian rhythm-mediated MTTP activation (Pan et al. 2010), regulating plasma triglyceride levels.

3.3 SHP and Glucose Metabolism

The fact that SHP serves as a co-regulator for a wide variety of NRs and non-NRs, some of which are involved in glucose homeostasis, suggests a role of SHP in regulating glucose metabolism. Indeed, bile acid-induced SHP represses hepatic gluconeogenic genes including Pck1, G6pc, and fructose 1,6-bis phosphatase (FBP1) by interfering the activity of HNF4 and forkhead box O (FoxO) 1 (Yamagata et al. 2004), transcription factors that upregulate the genes in the hepatic gluconeogenic program, while the bile acid-mediated inhibitory role of SHP in gluconeogenesis was lost in SHP-null mice (Ma et al. 2006). Additionally, studies have shown that SHP inhibits the activity of glucocorticoid receptor (GR) by antagonizing PGC-1α, which co-activates GR to upregulate Pck1 (Borgius et al. 2002). Similarly, SHP inhibits the activity of hepatocyte nuclear factor 3 (HNF3) (Kim et al. 2004) and CCAAT/enhancer-binding protein alpha (C/EBPα) (Park et al. 2007) to downregulate the hepatic gluconeogenic program.

4 Bile Acid-Activated Receptor Pregnane X Receptor (PXR, NR1I2)

The PXR functions as a xenobiotic sensor that is an essential component of the body's defense mechanism against toxic byproducts of endogenous metabolism and exogenous chemicals. It also functions as a receptor for endobiotics including bile acids to protect the body from their toxic effects. PXR was originally cloned from a mouse cDNA library in 1998 as an orphan NR activated by naturally occurring pregnanes and synthetic glucocorticoids and antiglucocorticoids (Kliewer et al. 1998). Subsequently, the human ortholog of PXR (referred as steroid and xenobiotic receptor, SXR, or pregnane-activated receptor, PAR) (Bertilsson et al. 1998; Blumberg et al. 1998) and rat PXR clones were isolated (Zhang et al. 1999). It has been shown that PXR is activated by structurally diverse ligands due to its unique characteristics of LBD, implying the promiscuity of the receptor function (Kliewer 2015; Watkins et al. 2001). Nevertheless, LCA was identified as a natural ligand for PXR (Staudinger et al. 2001) (Table 1). The expression of PXR is highly abundant in tissues exhibiting high metabolic activity including the intestine and liver with low expression levels in the kidney and stomach (Kliewer et al. 1998), suggesting its role in accommodating the high activity of metabolism and detoxification in those tissues.

Upon ligand binding, PXR binds as a heterodimer with RXR to xenobiotic response elements (XREs) composed of two half-sites organized as a direct repeat (DR) separated by various nucleotides including DR-3, DR-4, or DR-5, and an everted repeat (ER) with a 6 (ER-6) or 8 (ER-8) nucleotide spacer (Orans et al. 2005).

4.1 PXR and Bile Acid Metabolism

Consistent with the role of PXR as a xenobiotic receptor, PXR regulates transcription of genes in Phase I detoxification pathways including cytochrome p450 3A (CYP3A), CYP2B, and CYP2C; Phase II conjugation enzymes such as sulfotransferases (SULTs) and glutathione *S*-transferases (SULTs); and Phase III drug transporters such as MDR1, MRP2, and OATP2 (Kakizaki et al. 2008). Additionally, PXR regulates expression of genes involved in bile acid homeostasis to protect the liver from bile acid toxicity. Studies have shown that PXR is activated by LCA, a toxic hydrophobic secondary bile acid, and regulates genes involved in bile acid biosynthesis and transport such as Cyp7a1 and Oatp2, protecting liver damage induced by LCA (Staudinger et al. 2001). It was suggested that PXR induced by LCA upregulates CYP3A that mediates hydroxylation of bile acids, reducing the toxicity of LCA, which confers the protective effect of PXR on liver damage (Xie et al. 2001). Activation of PXR is also involved in bilirubin detoxification by upregulating the expression of the phase II UDP-glucuronosyltransferase family 1 member A1 (UGT1) (Xie et al. 2003), which promotes the conjugation of neurotoxic bilirubin to nontoxic bilirubin glucuronide.

4.2 PXR and Lipid Metabolism

Studies have shown that overexpression of the activated PXR specifically in the liver directly induces expression of cluster of differentiation 36 (CD36), a fatty acid translocase, along with other hepatic lipogenic genes such as SCD-1 and long-chain free fatty acid elongase, leading to an increase in hepatic triglyceride accumulation (Zhou et al. 2006). Interestingly, the increased hepatic TG accumulation is independent of SREBP-1c, a master transcriptional regulator of lipogenesis. Conversely, activation of PXR inhibits fatty acid β-oxidation and ketogenesis in the liver. It directly interacts with forkhead factor FoxA2 and represses its transcriptional activity that otherwise would upregulate expression of carnitine palmitoyltransferase 1A (Cpt1a) and mitochondrial 3-hydroxy-3-methylglutarate-CoA synthase 2 (Hmgcs2) (Nakamura et al. 2007), although the underlying mechanism of the inhibition remains elusive.

4.3 PXR and Glucose Metabolism

PXR downregulates hepatic gluconeogenesis. PXR in response to its ligand directly interacts with CREB and inhibits CREB's transcriptional activity that induces G6pc (Kodama et al. 2007). Consistently, transgenic mice expressing the activated PXR display reduced expression of genes in hepatic gluconeogenesis, including Pck1 and G6pc (Zhou et al. 2006). It has been shown that activation of PXR can function as a corepressor for FoxO1 by directly binding to FoxO1 to inhibit its binding to insulin-responsive elements (IREs) in the Pck1 promoter, downregulating FoxO1-mediated transcription of Pck1 upon activation by its ligand (Kodama et al. 2004). Additionally, a study has shown that activation of PXR leads to interfering with HNF4 to compete for a common coactivator, PGC-1α, which would result in inhibiting Pck1 transcription (Bhalla et al. 2004).

5 Bile Acids and Constitutive Androstane Receptor (CAR, NR1I3)

The CAR was originally cloned in 1994 from an adult human liver cDNA library as an orphan member of the NR superfamily (Baes et al. 1994). Subsequent studies isolated the mouse CAR clone and identified that CAR possesses constitutive transcriptional activity in the absence of its ligand (Choi et al. 1997). Interestingly, later, it was demonstrated that the constitutive transcriptional activity of CAR is reversed by androstane metabolites including androstanol and androstanol, which are considered naturally occurring ligands functioning as inverse agonists (Forman et al. 1998). In contrast to the inverse agonist, phenobarbital (PB) and 1,4-bis [2-(3, 5-dichloropyridyloxy)]benzene (TCPOBOP) further enhance the transcriptional activity of CAR, and TCPOBOP was identified as an agonist ligand for CAR (Honkakoski et al. 1998; Xie et al. 2000). For transcriptional activity, CAR

binds as a heterodimer partner with RXR to its specific DNA response elements composed of direct repeats of the half-sites separated by 5 (DR-5) or 3 (DR-3) nucleotides and inverted repeats with a 6 nucleotide spacer (IR-6) (Baes et al. 1994; Xie et al. 2000), and a distal 51-bp phenobarbital response element (PBRE) (Honkakoski et al. 1998). CAR is predominantly expressed in liver (Baes et al. 1994; Choi et al. 1997).

5.1 CAR and Bile Acid Metabolism

Similar to PXR, CAR has been established as a xenobiotic sensor. CAR induces CYPs genes that metabolize xenobiotics including human CYP2B6 and mouse Cyp2b10 and Cyp3A in response to PB and TCPOBOP, preventing the liver from the harmful effects of toxic compounds (Xie et al. 2000). However, unlike PXR, whether endogenous bile acids function as natural ligands for CAR remains elusive. Nevertheless, studies have shown that CAR plays a role in bile acid detoxification and clearance. Transgenic mice expressing the activated CAR exhibit resistance to LCA-mediated hepatotoxicity by inducing expression of genes such as SULT2A9 and 3′-phosphoadenosine-5′-phosphosulfate synthetase 2 (PAPSS2), which in turn promotes sulfation of LCA to detoxify it (Saini et al. 2004). Conversely, CAR deficiency leads to further exacerbation of liver damage in response to LCA by inhibiting the LCA-mediated induction of CYP3A11 and MRP3 (Zhang et al. 2004). Additionally, CAR knockout mice display more severe hepatic damage than wild-type mice, which is associated with alterations of expression of genes in bile acid synthesis and transporters such as Cyp7A1, Oatp-c, and sodium-dependent organic anion transporter 2 (Oatp2) (Stedman et al. 2005).

5.2 CAR and Lipid Metabolism

Studies have proposed a role of CAR in lipid homeostasis. Treatment of obese diabetic mice fed a high-fat diet with TCPOBOP, a CAR agonist, reverses the obese phenotypes and improves insulin sensitivity. CAR activation leads to a reduced hepatic lipogenic program, VLDL secretion, and export of triglycerides (Gao et al. 2009). Similarly, other study has shown that activation of CAR in response to TCPOBOP reduces mature SREBP-1 protein levels and its downstream lipogenic target, Scd-1, accompanied with reduced hepatic triglyceride levels. In line with the findings, the activation of CAR induces expression of insulin-induced gene 1 (Insig-1), a transmembrane protein localized in the endoplasmic reticulum (ER) where it binds to SREBP-1 blocking the translocation of mature SREBP-1 into the nucleus (Roth et al. 2008). Controversial studies, however, regarding the beneficial role of CAR in lipid homeostasis, have been reported. Activation of CAR upregulates expression of hepatic lipogenic genes such as Fasn and fatty acid elongase 6 (Elovl6), glycerol-3-phosphate acyltransferase, mitochondrial (Gpam), and thyroid hormone responsive (Thrsp, also known as Spot14), with concomitant increased

lipid accumulation in liver (Marmugi et al. 2016). CAR deficiency results in normalization of the high serum triglyceride levels in ob/ob mice, whereas TCPOBOP treatment induces serum triglyceride levels (Maglich et al. 2009).

5.3 CAR and Glucose Metabolism

An earlier study in type II diabetic patients has shown that chronic treatment of PB reduces blood glucose, increases glucose clearance rate, and improves insulin sensitivity (Lahtela et al. 1985), suggesting a role of PB in a glycemic control. Consistently, studies in rodents have shown that PB and TCPOBOP reduce the rate of hepatic glucose production and the activity of hepatic PCK1 and G6PC (Argaud et al. 1991; Manenti et al. 1987). Later studies have shown that CAR deficiency abrogates the reducing effects of PB and TCPOBOP on hepatic gluconeogenesis (Gao et al. 2009). Similar to PXR, CAR acts as a corepressor and directly interacts with FoxO1, which interferes with its binding to IREs in the Pck1 promoter, inhibiting the transcription of Pck1 (Kodama et al. 2004).

6 Bile Acid-Activated Receptor Vitamin D Receptor (VDR, NR1I1)

The VDR was originally cloned from human intestine and T47D cell cDNA libraries (Baker et al. 1988). Studies during the past two decades have established that calcitriol (1α,25-Dihydroxyvitamin D_3 ($1,25(OH)_2D_3$)), the active form of vitamin D, acts as an endogenous classic ligand for VDR, mediating several biological functions primarily associated with calcium homeostasis and skeletal maintenance (Brumbaugh and Haussler 1974). Notably, recent discoveries have demonstrated that secondary bile acids such as LCA and its metabolite 3-keto-LCA (Makishima et al. 2002), LCA acetate (Adachi et al. 2005), and LCA propionate (Ishizawa et al. 2008) act as natural ligands for VDR (Table 1), suggesting that VDR functions as a bile acid receptor. Unlike FXR, VDR is not activated by the primary bile acids, CDCA and CA, and the secondary bile acid DCA. Upon activation by its ligand, VDR interacts with its obligatory partner RXR and binds to its specific DNA elements consisting of direct repeats of half-sites separated by 3 nucleotides (DR-3) and everted repeats with 6 nucleotide spacer (ER-6) (Makishima et al. 2002). VDR is highly expressed throughout the digestion tract including duodenum, jejunum, ileum, and colon, along with other tissues such as the skin and kidney (Bookout et al. 2006). In the intestine, VDR in response to LCA induces expression of CYP3A that metabolizes toxic LCA (Makishima et al. 2002), possibly protecting the intestinal barrier function to prevent the overflow of LCA into the enterohepatic circulation and thus hepatotoxicity of LCA (Cheng et al. 2014). In the colon, MRP3, a multi-specific anion transporter for conjugated and unconjugated bile acids, is induced by VDR upon activation with LCA, but not in the liver, demonstrating a role of VDR in protecting colon cells from bile acid toxicity (McCarthy et al. 2005). Noticeably, hepatocytes in humans, rats, and

mice express very low levels of VDR mRNA and protein (Gascon-Barre et al. 2003), suggesting that VDR is more likely to exert its regulatory function in the intestine in response to LCA. Nevertheless, non-parenchymal cells including sinusoidal endothelial, Kupffer, and stellate cells express significant levels of functional VDR (Gascon-Barre et al. 2003). However, the biological function of VDR in non-parenchymal cells remains largely unresolved in regard to bile acid homeostasis.

7 Conclusions and Perspectives

Bile acids not only function as a facilitator to digest and absorb lipids and fat-soluble vitamins but also serve as signaling molecules that regulate many physiological functions. Bile acid homeostasis is maintained primarily by transcriptional regulation mediated by bile acid receptors, FXR, PXR, CAR, VDR, and SHP. Inter-organ communications mainly between the liver and the intestine are an integral part in maintaining bile acid homeostasis through the enterohepatic circulation (Li and Chiang 2014). As bile acids are integrated in regulating lipid, glucose, and energy metabolism, impaired regulation of bile acid homeostasis is associated with pathophysiology of metabolic disorders, including obesity, type 2 diabetes, and NASH. Progress has been made in recent years as to the development of drugs targeting the bile acid receptors to treat the metabolic diseases and cholestasis. OCA is the first FXR ligand that was approved by FDA to treat PBC, hepatic steatosis, inflammation, and fibrosis. Although the efficacy of OCA was proven, recent reports demonstrated side effects of OCA such as itching and a risk of liver injury, indicating that the future development of novel therapies are warranted (Adorini et al. 2012; Mudaliar et al. 2013; Pellicciari et al. 2002; Verbeke et al. 2016). Additionally, fexaramine, an intestinal-specific FXR agonist, improves metabolic abnormalities (Pathak et al. 2018); however, bile acid binding resins inhibiting FXR activity display similar effects (Smushkin et al. 2013). A systematic future research investigating the tissue-specific role of FXR in the intestine and liver would advance our understanding of the molecular action of FXR in the integrated metabolism and provides clues for the new development of pharmacological drugs. In addition to targeting FXR, further research to develop new agonists/antagonists for PXR, CAR, and VDR would also be anticipated.

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The Enterokine Fibroblast Growth Factor 15/19 in Bile Acid Metabolism

Marica Cariello, Marilidia Piglionica, Raffaella Maria Gadaleta, and Antonio Moschetta

Contents

Abstract

The endocrine fibroblast growth factors (FGFs), FGF19, FGF21, and FGF23, play a key role in whole-body homeostasis. In particular, FGF19 is a postprandial hormone regulating glucose homeostasis, glycogen and protein synthesis, and primary bile acid (BA) metabolism. In the ileum, BA-dependent farnesoid X receptor (FXR) activation induces the production of FGF19, which reaches the liver through the portal system where it represses the expression of CYP7A1, the rate-limiting enzyme of hepatic de novo BAs synthesis. Dysregulation of BA levels associated with alteration in FGF19 level has been depicted in different pathological conditions of the gut-liver axis. Furthermore, FGF19 exploits strong anti-cholestatic and anti-fibrotic activities in the liver. However, native FGF19 seems to retain peculiar hepatic pro-tumorigenic actions. Recently engineered FGF19 analogues have been recently synthetized, with fully retained BA

M. Cariello · M. Piglionica · R. M. Gadaleta
Department of Interdisciplinary Medicine, "Aldo Moro" University of Bari, Bari, Italy

A. Moschetta (✉)
Department of Interdisciplinary Medicine, "Aldo Moro" University of Bari, Bari, Italy

National Cancer Center, IRCCS Istituto Tumori "Giovanni Paolo II", Bari, Italy
e-mail: antonio.moschetta@uniba.it

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regulatory activity but without intrinsic pro-tumoral action, thus opening bona fide novel pharmacological strategy for the treatment of gut-liver axis diseases.

Keywords
Bile acid metabolism · Cholestasis · Fibroblast growth factors 19 · Hepatocellular carcinoma

1 FGF Family

The fibroblast growth factor (FGF) family comprises 22 members involved in a plethora of biological processes, including cell proliferation, differentiation and migration, angiogenesis, embryonic development, organogenesis, tissue injury repair, and metabolic regulation (Beenken and Mohammadi 2009). FGFs are grouped into seven subfamilies based on differences in sequence homology and phylogeny (Itoh and Ornitz 2004).

Human FGFs, which comprise ~150–300 amino acid residues, conserve ~120 amino acids with an identity of 30–60% and an overall amino acid identity of 56–71%. The homologous core region is characterized by 12 antiparallel β-strands flanked by divergent amino and carboxyl termini. The sequence variation of the N- and C-terminal tails of FGFs explains the different biology of the various members (Mohammadi et al. 2005).

Most of FGFs act as autocrine and paracrine factors and mediate their biological responses by binding tyrosine kinase FGF receptors (FGFR) on the cell surface. FGFRs, encoded by FGFR1–FGFR4 genes, contain an extracellular ligand-binding domain, a transmembrane domain, and a split intracellular tyrosine kinase domain (Gibbs et al. 2004; Ornitz 2000; Powers et al. 2000; Stathopoulos et al. 2004; Tassi et al. 2001). Typically, FGFs require an additional interaction with heparan sulfate glycosaminoglycans (HSGAGs) to activate their receptors. FGF binding to the FGFR and HSGAG induces receptor dimerization and the phosphorylation of specific tyrosine residues ultimately leading to the activation of cytoplasmic signal transduction pathways (Itoh and Ornitz 2008; Mohammadi et al. 2005; Ornitz et al. 1996; Ornitz 2000). The HSGAGs stabilize FGFs and prevent thermal denaturation and proteolysis and are required for the effective activation of FGFRs by FGFs (Ornitz 2000; Ornitz and Itoh 2001; Thornton and DeSalle 2000).

In contrast, the FGF15/FGF19 (FGF15 and FGF19 are the mouse and human orthologues, respectively), FGF21, and FGF23 subfamily display extremely low affinity to HSGAGs, thus acting as endocrine hormones. They are released into the bloodstream and are able to diffuse away from the tissues they are secreted from, exerting metabolic actions throughout the body (Goetz et al. 2007; Itoh 2010; Zhang et al. 2006). Different from the other FGFs, the endocrine FGF signal depends on single-pass transmembrane glycoproteins, named α-klotho and β-klotho, as co-receptors to bind and activate their cognate FGFRs (Kurosu et al. 2006; Ogawa et al. 2007; Urakawa et al. 2006; Wu et al. 2007). α-klotho acts as a co-receptor for FGF23, while β-klotho acts as co-receptors for FGF15/FGF19 and FGF21

(Kharitonenkov et al. 2008; Kurosu et al. 2006, 2007; Ogawa et al. 2007; Urakawa et al. 2006; Wu et al. 2007). Importantly, most tissues express different FGFR isoforms, while expression of klotho proteins is more restricted; thus, the presence or absence of the klotho proteins dictates the sites of action for these multitasking hormones (Adams et al. 2012; Ding et al. 2012; Kharitonenkov et al. 2008; Kurosu et al. 2007; Lin et al. 2007; Urakawa et al. 2006; Yang et al. 2012).

2 Endocrine FGFs

FGF15 has been the first endocrine FGFs to be identified and initially described as a downstream target of the chimeric homeodomain oncoprotein E2A-PBX1 in the brain (McWhirter et al. 1997). At the same time, in a screen to identify novel FGFs in the fetal brain, the human FGF19 gene was identified (Nishimura et al. 1999), and only later it has been found to be the human orthologue of mouse FGF15, given the synteny of the FGF15 and FGF19 genes (Katoh and Katoh 2003). Although FGF15 shares only 50% amino acid identity with its human orthologue FGF19 (McWhirter et al. 1997), instead of >90% as the most FGF, they have similar tissue expression profiles (Fon et al. 2010; Gimeno et al. 2002; Inagaki et al. 2005; Krejci et al. 2007; Nishimura et al. 1999; Xie et al. 1999) and comparable effects on gene expression elicited by their protein products in mice (Inagaki et al. 2005; Potthoff et al. 2011).

Homology-based PCR and analysis of the conserved amino acid core of mouse FGF15 have led to the identification of the other components of the endocrine FGF subfamily (Itoh and Ornitz 2004; Nishimura et al. 1999; Shimada et al. 2001; Yamashita et al. 2000). Following their identification and cloning, expression analysis has revealed different expression profile for each endocrine FGF and their role in bile acid (BA), cholesterol, glucose, vitamin D, and phosphate homeostasis (Fon et al. 2010).

FGF23 is the physiological regulator of phosphate and vitamin D serum level (Shimada et al. 2001, 2004a, b), and, in the parathyroid, it inhibits parathyroid hormone (PTH) secretion and contributes to its own negative feedback regulation (Ben-Dov et al. 2007; Galitzer et al. 2008; Lavi-Moshayoff et al. 2010). FGF23 is synthesized in the bone upon stimulation of mineral ions (phosphate and calcium) and vitamin D (Shimada et al. 2004a) and acts on its target organs via FGFR-α-klotho receptor complex (FGFR1c for the kidney and FGFR3c for the parathyroid gland) (Ben-Dov et al. 2007).

FGF21 exerts metabolic effects by acting as a hepatokine, adipokine, and myokine. Tissue-selective FGF21 signaling required β-klotho and one of the FGFRs, mostly FGFR1c (Ding et al. 2012; Kurosu et al. 2007; Ogawa et al. 2007). The main transcriptional inducers of FGF21 are fasting, ketogenic and high-carbohydrate diets, free fatty acids, and nuclear receptors (thyroid hormone receptor, retinoic X receptor β, farnesoid X receptor (FXR), retinoid-related orphan receptor α, nuclear hormone receptor 77, and peroxisome proliferator-activated receptor γ) (Badman et al. 2007; Inagaki et al. 2007; Lundasen et al. 2007), and it

has a role in the response to oxidative stress (Liu et al. 2013; Planavila et al. 2013, 2015) and regulation of glucose metabolism (Inagaki et al. 2007; Kharitonenkov and Shanafelt 2009; Potthoff et al. 2012), adaption to starvation (Inagaki et al. 2007, 2008; Potthoff et al. 2009; Zhang and Li 2014), regulation of thermogenesis, and WAT browning (Fisher et al. 2012; Holland et al. 2013; Hondares et al. 2011; Lin et al. 2013).

FGF15/FGF19 is predominantly expressed in the small intestine, gallbladder, brain, cartilage, skin, and kidney and is involved in a plethora of metabolic functions such as glucose homeostasis, glycogen synthesis, and bile acid (BAs) metabolism (Fon et al. 2010; Xie et al. 1999).

3 FGF15/FGF19 in Energy Homeostasis

Studies in FGF15- and FGFR4-knockout mice have uncovered the physiological role of this postprandial hormone in governing glucose homeostasis and glycogen and protein synthesis (Kir et al. 2011a; Potthoff et al. 2011).

Mice lacking FGF15 fail to properly maintain blood concentrations of glucose and show reduced hepatic glycogen. Pharmacological administration of FGF19 restores the normal regulation of glucose homeostasis and glycogen metabolism (Kir et al. 2011a). The regulation of hepatic carbohydrate homeostasis by FGF19 depends on the inhibition of hepatic gluconeogenesis through a mechanism involving the dephosphorylation and inactivation of the transcription factor cAMP regulatory element-binding protein (CREB) (Potthoff et al. 2011). Mice with constitutively elevated levels of FGF19 have lower body weight due to reduced fat content, despite having elevated food intake, and they are protected from diet-induced obesity (Tomlinson et al. 2002). Furthermore, the elevated energy expenditure resulting from increased hepatic fatty acid oxidation (via repression of acetyl-CoA carboxylase 2, ACC2, and stearoyl-CoA desaturase 1, SCD1) explains the reduced adiposity and the increased food intake (Fu et al. 2004; Tomlinson et al. 2002). Interestingly, administration of FGF19 in Fgfr4-KO mice on a high-fat diet improves glucose homeostasis, demonstrating that FGF19 does not require FGFR4 binding in order to mediate these effects (Wu et al. 2011).

In addition to glucose homeostasis and glycogen metabolism, FGF19 also activates the hepatic protein synthesis machinery in vivo, using a RAS-ERK-p90RSK pathway to induce ribosomal protein S6 phosphorylation (Kir et al. 2011a).

4 Role of FGF15/FGF19 in the Gut-Liver Axis

Bile acids (BAs) are strong detergent molecules synthesized in the liver. Upon secretion into the hepatic canalicular membrane, they are stored in the gallbladder until the pancreatic hormone colecistokinine is released upon food ingestion. Subsequently, BAs are released into the small intestine where they start their journey

aimed to aid the digestion and absorption of lipids and fat-soluble vitamins. In the terminal ileum, 95% of BAs are reabsorbed and returned to the liver, where they are ready to be resecreted in a cycle known as enterohepatic circulation. BAs exert several metabolic functions: they contribute to cholesterol catabolism and prevent gut bacterial overgrowth, intestinal injury, and cholestasis (Choi et al. 2006). Given their intrinsic detergent nature, elevated BA concentration is toxic for the organism; therefore, their levels must be tightly regulated.

FXR is the master regulator of BA homeostasis (Calkin and Tontonoz 2012; de Aguiar Vallim et al. 2013; Matsubara et al. 2013). It is mainly express in the intestine and the liver exploiting in both organs a synergistic action leading to the repression of cholesterol 7α-hydroxylase (Cyp7a1), the rate-limiting enzyme that converts cholesterol into BAs in the liver. Cyp7a1 expression is regulated by the hepatic small heterodimer partner (SHP), an orphan nuclear receptor (Goodwin et al. 2000; Lu et al. 2000). SHP is recruited through the interaction of LRH-1 and HNF4α (Chiang and Stroup 1994; De et al. 2001; Goodwin et al. 2000; Lu et al. 2000). In fact, Shp−/− mice present with elevated basal Cyp7a1 expression (Kerr et al. 2002; Wang et al. 2002). The intestinal FGF15/FGF19-FGFR4 pathway synergizes with the hepatic SHP pathway to repress cyp7a1 expression via the Jun N-terminal kinase-dependent pathway (Inagaki et al. 2005). In the ileum, a functional farnesoid X receptor responsive element (FXRE) within the second intron of FGF19 has been identified (Holt et al. 2003). In fact, FXR induces the transcription of the fibroblast growth factor FGF15/FGF19, which is then secreted into the portal circulation and reaches the liver, where it binds to FGFR4-β-klotho, initiating a phosphorylation cascade pathway (Holt et al. 2003), ultimately repressing the transcription of Cyp7a1 (Inagaki et al. 2005) (Fig. 1). Both Fgf15-KO (Inagaki et al. 2005) and intestine-specific (but not liver-specific) Fxr-KO mice (Kim et al. 2007) fail in FXR-mediated repression of CYP7A1 when treated with FXR agonists, demonstrating that activation of the intestinal Fxr-Fgf15 duo is necessary for Cyp7a1 repression in the liver. To carry out its function, FGF15/FGF19 also has to bind the receptor FGFR4 coupled with the co-receptor β-klotho triggering the downstream signaling cascades (Cicione et al. 2012; Degirolamo et al. 2016; Lin et al. 2007). Several downstream pathways, including extracellular signal-regulated kinase 1 (ERK1), ERK2, RAS–ERK–p90, and dephosphorylation and inactivation of cAMP-response element-binding protein (CREB), mediate the actions of the FGF19–FGFR4–β-klotho complex (Inagaki et al. 2005; Kir et al. 2011a; Kong et al. 2012). It has been demonstrated that the C-terminus region is responsible for the binding to β-klotho, whereas the N-terminus seems important for FGFR activation (Wu et al. 2008). Interestingly, studies conducted on Fgfr4−/−, Fgf15−/−, and β-klotho−/− mice have demonstrated that these mice exhibit upregulated Cyp7a1 and elevated BAs levels (Inagaki et al. 2005; Ito et al. 2005; Yu et al. 2000) even upon administration of GW4064 or cholic acid. Although FGF15/FGF19 transcriptional regulation has been unraveled, the factors driving FGF19 secretion in enterocytes are still unclear. A recent study has identified Diet1, an intestinal factor that influences FGF15/FGF19 expression at a posttranscriptional level in mouse intestine and human

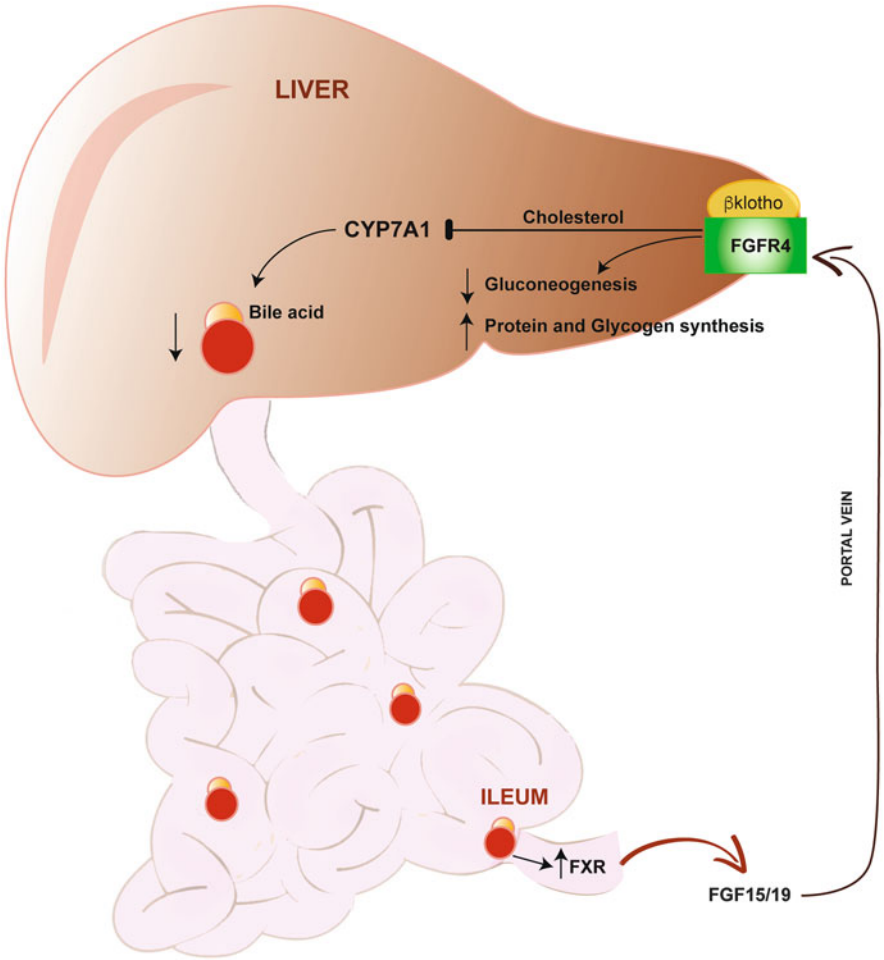

Fig. 1 FGF19 in the gut-liver axis. BA-dependent FXR activation induces the secretion in the portal circulation of the FGF15/FGF19 that reaches the liver and binds the receptor FGFR4 with the co-receptor β-klotho, repressing CYP7A1 expression, thus reducing BAs synthesis. In the liver, FGF19 reduces gluconeogenesis and promotes protein and glycogen synthesis

enterocytes (Reue et al. 2014). Diet1 is a determinant of FGF15/FGF19 secretion by the enterocytes, with downstream effects on hepatic BA synthesis and cholesterol levels.

Further evidence generated by clinical studies supports the importance of FGF19 role in BAs homeostasis. Serum FGF19 levels have a diurnal rhythm with peaks after postprandial stimulus. This peak is associated with the repression of BAs synthesis (Lundasen et al. 2006). In humans, oral administration of bile acids

increases serum FGF19 levels, and cholestyramine administration, a bile acid sequestrant, reduced circulating FGF19 levels (Lundasen et al. 2006). Its serum levels are also reduced in patients who have undergone ileal resection or Crohn's disease (Cho et al. 2010). Moreover, a clinical study demonstrated that patients with primary and secondary bile acid diarrhea have reduced FGF19 levels compared to healthy subjects leading to increased BA synthesis and diarrhea (Pattni and Walters 2009; Pattni et al. 2013). Additionally, severe diarrhea resulted in monkeys when FGF19 was neutralized with antibodies (Reue et al. 2014).

The expression of FGF15 in mice is induced by other nuclear receptors, such as the vitamin D receptor (VDR), retinoid X receptor (NR2B1), and pregnane X receptor (NR1I2) (Schmidt et al. 2010; Wistuba et al. 2007), and by a chronic high-cholesterol diet (Henkel et al. 2011).

Associated with inhibition of BA synthesis, FGF15/FGF19 also plays an important role in the control of gallbladder volume. FGF15/FGF19 promotes gallbladder filling, action partly mediated via relaxation of the gallbladder smooth muscle (Choi et al. 2006). Concomitantly, BAs released in the small intestine inhibit cholecystokinin secretion, which in turn blocks gallbladder contraction. Fgfr4−/−, Fgf15−/−, and β-klotho−/− mice exhibited a smaller gallbladder volume that it restored to normal parameters in Fgf15−/− mice after FGF15 administration (Choi et al. 2006; Kir et al. 2011b). These findings suggest that FGF15/FGF19 regulates bile acid homeostasis, including the postprandial refilling of the gallbladder.

5 FGF19 and Hepatic Diseases

Dysregulation of BA levels has been described in different diseases of the gut-liver axis. Therefore, it is not surprising that alterations of FGF19 levels may concur to the onset and development of hepatic disorders ranging from cholestasis to fibrosis and hepatocellular carcinoma (HCC).

Cholestasis is defined as any medical condition characterized by acute or chronic impairment of bile flow with hepatic accumulation of BAs and xenobiotics that play a key role in cholestasis-associated liver damage. The etiology of cholestasis can be diverse. Extrahepatic cholestasis can be ascribable to bile duct tumors, cysts, and stones in the bile duct, whereas intrahepatic cholestasis can be triggered by sepsis, drugs, primary biliary cirrhosis (PBC), primary sclerosing cholangitis (PSC), viral hepatitis, alcoholic liver disease, and pregnancy. Five multiple heritable forms of progressive familial intrahepatic cholestasis (PFIC) characterized by mutations in ATPase phospholipid transporting 8B1(ATP8B1), in the canalicular bile salt export pump (BSEP) gene, in the canalicular phospholipid flippase multidrug resistant protein 3 (MDR3), in the tight junction protein 2 (TJP2), and in the nuclear receptor FXR genes have been described (Bull et al. 1998; Davit-Spraul et al. 2014; Gomez-Ospina et al. 2016; Jacquemin et al. 2001; Sambrotta et al. 2014).

Hepatic accumulation of BAs plays a central role in the pathogenesis of cholestasis-induced liver injury, and the reduction of hepatic BA is essential to prevent liver damage and disease progression (Hirschfield et al. 2010). Modica et al. demonstrated that in basal condition, wild-type mice treated with FGF19 for 5 days presented with a 30% reduction of the total BA pool size and enrichment in TβMCA, a hydrophilic BA (Modica et al. 2012). Furthermore, in wild-type mice treated with FGF19 under bile duct ligation (BDL), a reduction in liver inflammation and necrosis was observed (Modica et al. 2012), demonstrating that FGF19 protected against cholestasis through the reduction of the total BA pool size. To date, liver transplantation is the available therapy for late-stage cholestasis, while ursodeoxycholic acid (UDCA) is the only approved drug for the treatment of this disease (Lindor 2007). In this scenario, FGF19-based therapy could represent a novel potential therapeutic approach. However, safety concerns have been raised due to the known mitogenic properties of FGF19 (Latasa et al. 2012; Nicholes et al. 2002; Sawey et al. 2011; Schaap et al. 2009), and whether FGF19 causes (Nicholes et al. 2002) or not (Avila and Moschetta 2015; Naugler et al. 2015) HCC is still unclear. FGF19 is overexpressed in HCC patients and correlates with worse prognosis (Miura et al. 2012). In a transgenic mouse model, overexpression of human FGF19 in skeletal muscle promotes hepatocyte proliferation and HCC development (Nicholes et al. 2002). Furthermore, FGF19 transgenic mice develop HCC between 10 and 12 months of age and exhibit nuclear accumulation of β-catenin (Nicholes et al. 2002). The tumorigenic activity of FGF19 has been ascribed to a cross talk between FGFR4 and β-catenin (French et al. 2012). FGF19 and FGFR4 are co-expressed in human liver, lung, and colon tumors (Desnoyers et al. 2008). In mice with ectopic FGF19 expression, the use of FGFR4-neutralizing antibody was able to suppress tumor growth suggesting the possibility to modulate FGF19-mediated hepatocyte proliferation (French et al. 2012; Pai et al. 2008). In addition, hepatocyte-specific deletion of stat3 and genetic or pharmacological ablation of il6 blocked FGF-19-induced HCC retaining the FGF19 beneficial functions on BA, glucose, and energy metabolism (Zhou et al. 2017). In line with this, the removal of the domain responsible for FGFR4 binding allowed to preserve the FGF19 helpful effects (Wu et al. 2010, 2011). To overcome this major drawback due to the unclear tumorigenic property of FGF19, Zhou et al. generated a non-tumorigenic engineered variant of FGF19, named FGF19-M70, differing from wild-type FGF19 by 5-amino acid deletion (P24-S28) and the substitution of three amino acids (A30S, G31S, H33L) at the N-terminus (Zhou et al. 2014). In mouse models of obstructive extrahepatic and intrahepatic cholestasis (BDL and ANIT, respectively), M70 administration modulated FGFR4 signaling to suppress hepatic Cyp7a1 expression reducing hepatic BA concentration and serum liver enzymes leading to protection from cholestatic liver injury (Luo et al. 2014). In a mouse model of familial intrahepatic cholestasis, M70 reduced liver injury, hepatic inflammation, and biliary fibrosis inhibiting Cyp7a1 expression and decreasing BA pool size. FGF19-M70 is able to activate only the subset of signaling pathways downstream of FGFR4,

preserving BA regulatory function while losing the Stat-3-dependent pro-tumoral activity (Zhou et al. 2014). Moreover, in a mouse model of NASH, FGF19-M70 reduced liver injury and ameliorated histological features of NASH, glucose, and lipid metabolism representing a promising approach to treat the disease (Zhou et al. 2016). Unlike FGF19, M70 did not cause liver tumors after injection in db/db mice. In fact, in this model the expression of markers including glutamine synthase, Ki-67, α-fetoprotein, and cyclins is increased by FGF19 but not by FGF19-M70 administration.

Recently, it has been demonstrated that circulating levels of FGF19, leading to the inhibition of BA synthesis, are proportionally increased in response to the severity of cholestasis in patients with PBC (Li et al. 2017). FGF19 levels also correlate with Mayo Risk Score, a model for a short-term survival probability of PBC patients (Wunsch et al. 2015). In these patients, FGF19-mediated suppression of BA synthesis is unable to reverse disease progression, and pharmacological intervention with FGF19 analogues could represent a novel approach to treat cholestasis.

Given its therapeutic role in cholestatic liver disease, the therapeutic potential of FGF19 analogues could be extended to the treatment of HCC. FGF19 levels decreased in patients with primary BA malabsorption promoting an increase of BA synthesis and diarrhea (Walters et al. 2009). Moreover, patients with inflammatory bowel disease with resection of the distal ileum showed elevated serum BA levels and reduced FGF19 expression (Lenicek et al. 2011).

Very recently, it has been demonstrated that a novel non-tumorigenic analogue, namely, FGF19-M52, inhibits Cyp7a1, thereby reducing the circulating BA pool size and shifting its composition to a more hydrophilic one. This results in a significant reduction of biochemical parameters of liver damage and reduced expression of several genes driving the proliferative and inflammatory hepatic scenario, protecting Abcb4−/− and Fxr−/− mice against BA-induced spontaneous hepatocarcinogenesis (Gadaleta et al. 2018). Further studies are needed in order to translate the therapeutic potential of FGF19 analogue administration in clinical settings of cholestasis and HCC.

6 FGF19-Based Therapy: Promising Reality

FGF19 is emerging as an intestinal hormone with pleiotropic array of metabolic actions connecting the gut, the liver, the adipose tissues, and the central nervous system. Given its role of FGF19 in BA homeostasis and glucose metabolism, several clinical trials are ongoing exploiting FGF19 biological activities in the treatment of gut-liver axis diseases.

FGF19 exerts potent antidiabetic effects in mouse models (Fu et al. 2004; Morton et al. 2013). Morton et al. have evaluated the effects of carbohydrates, proteins, or

lipids on FGF19 levels in human samples. The authors have demonstrated that carbohydrate ingestion increased FGF19 levels, whereas lipid administration has a little influence on this hormone (Morton et al. 2014). Furthermore, Gomez-Ambrosi et al. analyzed the impact of weight loss induced by conventional dietary treatment or bariatric surgery on FGF19 concentration in 137 obese patients and 33 lean subjects (Gomez-Ambrosi et al. 2017). FGF19 levels were reduced in obese patients and increased after surgically induced weight loss indicating that low FGF19 levels are associated to visceral adiposity (Gomez-Ambrosi et al. 2017). Similarly, Roux-en-Y gastric bypass surgery in obese patients restored low circulating FGF19 levels to normal levels (Pournaras et al. 2012). Further studies are needed to analyze the therapeutic potential of FGF19 in the treatment of obesity and type II diabetes.

As shown in Table 1, several clinical trials have been carried on to evaluate the effects of FGFR inhibitors on solid tumors. The imbalance of the FGFR signaling has been linked to human tumors like gastric, breast, endometrial, bladder, and lung cancer and cholangiocarcinoma (Helsten et al. 2015; Wu et al. 2013). ARQ087 is an adenosine triphosphate (ATP)-competitive pan-FGFR inhibitor with multi-kinase activity. A phase I study has demonstrated that ARQ087 was well tolerated and it showed antitumor activity in patients with specific FGFR genetic alterations (Papadopoulos et al. 2017).

The discovery of M70, which fully retains BA regulatory activity but is devoid of pro-tumoral activity in mouse models (Zhou et al. 2014), has opened novel pharmacological strategy for the treatment of gut-liver axis diseases. To date, NGM282 (i.e., the FGF19-M70) is the only FGF19 analogue to enter in clinical studies (Table 1). In a randomized trial in healthy human volunteers, the administration of FGF19-M70 for 7 days (3 mg/day) was well tolerated and produced a reduction of C4 (a marker of Cyp7a1 activity) and postprandial total BAs serum levels, indicating that FGF19 has a prominent role in the regulation of BA synthesis and suggesting that the M70 analogue could offer an effective solution in the treatment of cholestasis in humans (Luo et al. 2014).

Very recently, a phase II clinical trial has evaluated the safety and efficacy of NGM282 for the treatment of NASH. In these patients, the administration of 3 mg or 6 mg of NGM282 was well tolerated and reduced liver fat content as well as liver inflammation and fibrosis (Harrison et al. 2018). Also, in the phase II clinical trial, the effects of NGM282 were studied on gastric and colonic transit, stool frequency, and consistency in patients with functional constipation (Oduyebo et al. 2018). The administration of NGM282 (1 and 6 mg/day) for 14 days was able to accelerate colonic transit increasing bowel movements and loosening stool consistency. The other adverse events were injection site reaction and diarrhea (Oduyebo et al. 2018).

The completion of the ongoing clinical trials and further studies are needed to evaluate FGF19 therapeutic potential in the management diseases of unmet medical needs, such as cholestasis and NASH, but also eventually type II diabetes and obesity.

Table 1 FGFR inhibitors and FGF19 mimetics in clinical trials

Trial identifier	Trial phase (status)	Study title	Disease	Intervention
FGFR inhibitor				
NCT02508467	Recruiting	A phase 1 study of BLU-554 in patients with hepatocellular carcinoma	HCC	BLU-554
NCT02325739	Recruiting	FGF401 in HCC and solid tumors characterized by positive FGFR4 and KLB expression	HCC	FGF401 and PDR001
NCT02808312	Recruiting	Pharmacokinetics and pharmacodynamics of GS-9674 in adults with normal and impaired hepatic function	NASH	GS-9674
NCT03144661	Recruiting	An open-label safety and tolerability study of INCB062079 in subjects with advanced hepatocellular carcinoma and other malignancies	HCC Cholangiocarcinoma Esophageal cancer	INCB062079
NCT02421185	Recruiting	Study to evaluate the safety, pharmacokinetics, and pharmacodynamics of JNJ-42756493 (erdafitinib) in participants with advanced hepatocellular carcinoma	HCC	JNJ-42756493 (erdafitinib)
NCT02038673	Completed	An open-label phase I study of orally available novel small-molecule fibroblast growth factor receptors (FGFR) 1,2,3, and 4 inhibitor, ASP5878 at single and multiple doses in patients with solid tumors	Solid tumors	ASP5878

(continued)

Table 1 (continued)

Trial identifier	Trial phase (status)	Study title	Disease	Intervention
NCT01948297	Recruiting	Debio 1347-101 phase I trial in advanced solid tumors with fibroblast growth factor receptor (FGFR) alterations	Solid tumors	Debio1347 (CH5183284)
NCT02834780	Recruiting	Phase 1 study to evaluate the safety, pharmacokinetics, and pharmacodynamics of H3B-6,527 in subjects with advanced hepatocellular carcinoma	Advanced HCC, intrahepatic cholangiocarcinoma	H3B-6527
NCT02592785	Completed	Phase I dose escalation study of BAY 1163877 (rogaratinib) in Japanese subjects with refractory, locally advanced, or metastatic solid tumors	Neoplasms	BAY1163877
NTC01752920	Active, not recruiting	Phase 1/2 study of ARQ 087 in adult subjects with advanced solid tumors with FGFR genetic alterations	Solid tumors	ARQ087
FGF19 mimetic				
NCT02135536	Completed	Phase 2b study of NGM282 extended treatment in patients with primary biliary cirrhosis	PBC	NGM282
NCT02649062	Completed	Study of NGM282 in subjects with functional constipation and healthy individuals	Functional constipation	NGM282
NCT02704364	Completed	Phase 2 study of NGM282 in patients with primary sclerosing cholangitis	PSC	NGM282

(continued)

Table 1 (continued)

Trial identifier	Trial phase (status)	Study title	Disease	Intervention
NCT02026401	Completed	Phase 2 study of NGM282 in patients with primary biliary cirrhosis	PBC	NGM282
NCT02443116	Recruiting	Study of NGM282 in patients with nonalcoholic steatohepatitis (NASH)	NASH	NGM282
NCT01943045	Completed	Phase 2 study of NGM282 in patients with type 2 diabetes	Diabetes mellitus	NGM282
NCT01776528	Completed	Phase 1 SAD and MAD study of NGM282 in healthy adult participants	Diabetes mellitus	NGM282

NAFLD nonalcoholic fatty liver disease, *MS* metabolic syndrome, *NASH* nonalcoholic steatohepatitis, *PBC* primary biliary cirrhosis, *PSC* primary sclerosing cholangitis

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Signaling from Intestine to the Host: How Bile Acids Regulate Intestinal and Liver Immunity

Michele Biagioli and Adriana Carino

Contents

Abstract

Primary bile acids (BAs) are generated in the liver as the end products of cholesterol catabolism; they are then conjugated and accumulated in the gallbladder. After a meal ingestion, BAs are reversed into the duodenum to facilitate the lipid absorption. At the intestinal level, the 95% of BAs are reabsorbed and redirected into enterohepatic circulation; indeed only a small amount of them are then subjected to chemical modifications by the intestinal microbiota, which plays a very important role in the generation of secondary bile acids and in regulating host's metabolism and activity of the immune system. Behind their role in nutrients absorption, bile acids act as signaling molecules, activating several receptors, known as bile acid-activated receptors (BARs), including the farnesoid-X-receptor (FXR) and the G protein-coupled bile acid receptor 1 (GPBAR1 or Takeda G-protein receptor 5). Both receptors appear to contribute to maintain the tolerogenic state of the liver and intestine immunity. In particular, FXR and GPBAR1 are highly expressed in cells of innate immunity including intestinal and liver macrophages, dendritic cells, and natural killer T cells. In this chapter, we provide an overview on mechanisms through which FXR and

M. Biagioli (✉) · A. Carino
Department of Surgical and Biomedical Sciences, University of Perugia, Perugia, Italy
e-mail: michele.biagioli@unipg.it; adriana.carino@unipg.it

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GPBAR1 modulate the signaling between microbiota and intestinal and liver innate immune system. This overview could help to explain beneficial effects exerted by GPBAR1 and FXR agonists in the treatment of metabolic and immuno-mediated diseases.

Keywords
Bile acids · Farnesoid-X-receptor · G-protein bile acid receptor · Immune system · Inflammation · Microbiota

1 Bile Acids and Intestinal Microbiota

Chemical diversity of bile acid pool is generated via subsequent host and bacterial modifications of primary bile acids CA and CDCA. Prior to secretion into bile, CA and CDCA are conjugated to glycine or taurine (Chiang 2009; Falany et al. 1994). The conjugation step is regulated by the bile acyl-CoA synthetase and bile acid-CoA amino acid *N*-acyltransferase (BAAT) for tauro- and glycine-conjugation, respectively. In humans, conjugation of primary bile acids with glycine and taurine follows a ratio of ≈3:1, giving rise to conjugated primary bile acids, i.e., glyco-CA (GCA) and glyco-CDCA (GCDCA), and tauro-CA (TCA) and tauro-CDCA (TCDCA). In contrast to human, in mice, the large majority of bile acids (>95%) are tauro-conjugated. It should be emphasized that conjugation of CA and CDCA with glycine and taurine increases their water solubility, but has no effect on the affinity of bile acids with their receptors. Bile acids produced in hepatocytes are then accumulated in the gallbladder and subsequently released into the duodenum after the ingestion of a meal, to facilitate absorption of triglycerides, cholesterol, and lipid-soluble vitamins (Chiang 2009). At the intestinal level, bile acids are effectively reabsorbed (>95%) mostly through active transport mediated by the ileal bile acid transporter (IBAT; also known as ASBT or SLC10A2). A contribution to bile acids resorption is also provided by passive diffusion into the upper small intestine and colon (Makishima et al. 1999; Grober et al. 1999).

In the intestine, the complex metabolism of primary bile acids is largely modulated by intestinal microbiota by a bidirectional manner, because conjugated bile acids are then subjected to chemical modifications by the microbiota (Wang et al. 2002). In the distal ileum, glyco- and tauro-CA and -CDCA are metabolized by the intestinal microbiota to generate secondary bile acids (Fiorucci and Distrutti 2015). The first step involves the deamination on the side chain performed by the microbial bile salt hydrolases (BSH), an enzyme expressed mostly by anaerobic intestinal bacteria belonging to the genera *Bacteroides*, *Clostridium*, *Lactobacillus*, and *Bifidobacteria*, followed by the 7α-dehydroxylation on the ring B made by a bacterial 7α-dehydroxylase, mainly expressed by *Clostridium* and *Eubacterium*. These reactions convert CA in deoxycholic acid (DCA) and CDCA in lithocholic acid (LCA). DCA and LCA are secondary (or degenerated) bile acids (Fig. 1). Therefore, the intestinal microbiota plays a very important role in the generation of secondary bile acids and, by modifying the composition of the bile acid pool, it

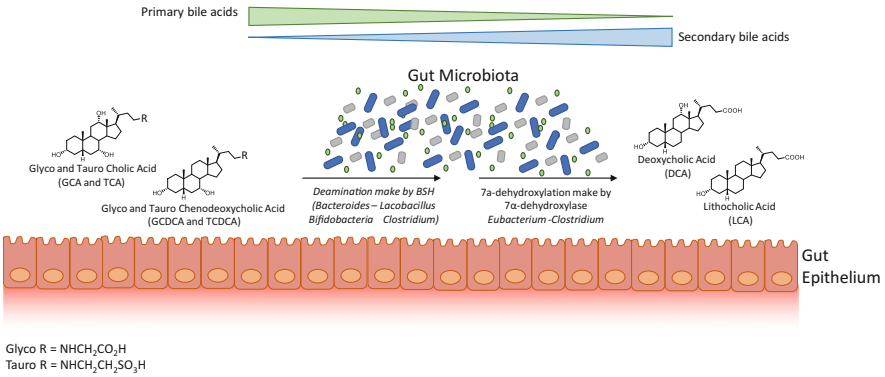

Fig. 1 Primary bile acids, CA and CDCA, are synthesized in the liver from cholesterol, conjugated and then secreted into bile ducts and transported to the intestine after the ingestion of a meal, to facilitate the lipid absorption. At the intestinal level, the 95% of BAs are reabsorbed and redirected into enterohepatic circulation; the remaining 5% of primary BAs pool, in the distal ileum, are largely modulated by intestinal microbiota to generate secondary BAs. The first step involves the deamination on the side chain performed by the microbial bile salt hydrolases (BSH), an enzyme expressed predominantly by *Bacteroides*, *Clostridium*, *Lactobacillus*, and *Bifidobacteria*, followed by the 7α-dehydroxylation on the ring B made by a bacterial 7α-dehydroxylase, mainly expressed by *Clostridium* and *Eubacterium.* These reactions convert CA in deoxycholic acid (DCA) and CDCA in lithocholic acid (LCA)

has an essential role in regulating many host's processes, i.e., metabolism and activity of the immune system (Ridlon et al. 2006; Fiorucci et al. 2018a).

The size and the composition of the bile acid pool are very important for the regulation of intestinal microbiota. Bile acids in a direct way, acting as antimicrobials (Inagaki et al. 2006), or indirectly through the induction of the production of antimicrobial peptides through FXR or by regulating the host's immune system through both FXR and GPBAR1 (Vavassori et al. 2009; Cipriani et al. 2011), are able to modify the structure of the intestinal microbiota. In summary, low levels of bile acids in the intestine favor the expansion of Gram-negative members of the intestinal microbiota which also include many potential pathogens. On the other hand, high levels of bile acids in the gut promote Gram-positive growth (Islam et al. 2011).

2 Bile Acids and Immune System

BARs play important roles in immune regulation. FXR and GPBAR1 are found in non-epithelial cells, including intestinal muscles and neurons (GPBAR1), biliary cells (FXR and GPBAR1), liver sinusoidal cells, and intestinal and liver endothelial cells (FXR and GPBAR1) (Fiorucci and Distrutti 2015). Furthermore, both receptors, as well as VDR and LXRs, are expressed at high concentrations in immune cells such as monocytes, macrophages, dendritic cells (DCs), natural killer

(NK) (ILC1) and NKT cells, while T and B lymphocytes express these receptors at low levels (Vavassori et al. 2009; Cipriani et al. 2011; Islam et al. 2011; Maruyama et al. 2002; Mencarelli et al. 2009; Biagioli et al. 2017). Activation of BARs in macrophages, DCs, and NKT results in several regulatory functions that are inhibitory in nature, and both receptors appear to contribute to maintain the tolerogenic state of the liver and intestine innate immunity (Chiang 2009).

2.1 Role of Bile Acids in the Regulation of Intestinal and Liver Immunity

Inflammatory bowel diseases (IBDs) are characterized by chronic inflammation of the intestinal tract associated with an imbalance of the intestinal microbiota. The prevalence of IBDs, specifically ulcerative colitis and Crohn's disease, has increased greatly over the past decades. Recent studies have pointed out genes associated with IBDs susceptibility that, together with environment factors, may contribute to the outcome of the disease. The exact etiology of IBDs remains largely unknown and treatment has focused on controlling inflammation. Many studies have been carried out to investigate the role of bile acids and BARs, especially GPBAR1 and FXR, in the regulation of intestinal immunity and microbiota homeostasis, giving substance to the idea that modulation of bile acid signaling may actually be beneficial in the context of IBDs.

Some evidence indicates that intestinal bile acid deficiency, due for example to bile duct obstruction, is associated with bacterial overgrowth and translocation, and intestinal mucosal damage. It is now known that the administration of bile acids or synthetic compounds such as GW4064, a selective FXR agonist, is able to control bacterial growth and translocation in the intestine (Inagaki et al. 2006; Ding et al. 1993).

Furthermore, two independent studies by Vavassori et al. (2009) and Gadaleta et al. (2011) reported that FXR activation in mice with obeticholic acid (OCA) decreases the severity of colitis induced by dextran sodium sulfate (DSS) or trinitrobenzenesulfonic acid (TNBS), maintaining intestinal epithelial barrier integrity and decreasing pro-inflammatory cytokines production, acting directly on the cells of the immune system (Vavassori et al. 2009; Gadaleta et al. 2011). These data indicate that FXR may be considered a therapeutic target for the treatment of IBDs, but clinical trials are not in progress probably due to the lack of molecules with a restricted action to the intestine (Massafra et al. 2018; Donkers et al. 2018).

Moreover, the study of GPBAR1 functions at intestinal level has shown that this receptor plays a protective role in the intestine by attenuating inflammatory processes. Indeed Gpbar1$^{-/-}$ mice show a greater intestinal permeability than wild type (Cipriani et al. 2011). In addition, it was shown that the activation of GPBAR1 by various compounds, 3-aryl-4-isoxazolecarboxamide (Yoneno et al. 2013), BAR501 (Biagioli et al. 2017), Betulinic acid (Sakanaka et al. 2015), oleanoic acid (Cipriani et al. 2011), and BIX02694 (Fryer et al. 2014), attenuates the gravity of mouse models of colitis and the production of pro-inflammatory cytokines. In conclusion,

there is sufficient preclinical evidence to show that the activation of GPBAR1 can have beneficial effects in the treatment of IBDs, but at the moment there are no clinical trials in progress.

The liver is the major regulator of organism metabolism, involved in nutrient deposition and detoxification from foreign substances. Furthermore, the liver protects the organism from invasion of dietary products and commensal bacterial molecules gut-derived, potentially inflammatory, by producing many components of immune system, including complement components, acute phase proteins, cytokines, and chemokines (Crispe 2009). As a result, a central organ of the immune system containing several populations of resident myeloid and lymphoid immune cells can be considered, which play an important role in the hepatic immune homeostasis, as well as in the recognition and effector response to invading toxic products, malignant cells, or pathogens (Crispe et al. 2006). In physiological conditions, the liver homeostasis is guaranteed by different mechanisms, such as immune and endotoxin tolerance, and control of persistent inflammation. When invading pathogens reach the liver from gut, via biliary epithelium or from systemic circulation, a rapid activation of the hepatic immune system occurs, to eliminate the harmful stimuli or resolve inflammation (Robinson et al. 2016). The bile acids are well known for their anti-inflammatory properties and play a fundamental role in the regulation of hepatic innate immune responses (Schubert et al. 2017).

Clinical observations in patients affected from hepatobiliary diseases suggest that bile acids inhibit cell-mediated immunity and macrophage activation (Swann et al. 2011; Keane et al. 1984; Fiorucci et al. 2010). As described above, all bile acids act also as signaling molecules, by activating the bile acids-activated receptors (BARs) (Chiang 2009). In the liver the hepatocytes express FXR, but not GPBAR1; conversely, both receptors were found in non-parenchymal cells such as biliary cells, liver endothelial and sinusoidal cells (Fiorucci and Distrutti 2015). Interestingly, both FXR and GPBAR1 are expressed in innate immune cells, including monocytes and macrophages, dendritic cells (DCs), natural killer (NK) (ILC1) and NKT cells (Maruyama et al. 2002; Mencarelli et al. 2009). In cells of adaptive immunity, T and B cells, we found a lower level of expression of these receptors. The BARs activation in macrophages, DCs, and NKT contributes to maintain the tolerogenic state of the liver innate immunity (Fiorucci et al. 2018a).

It has been reported that FXR null mice shows marked liver injury and increased hepatic expression of inflammatory genes (Kim et al. 2007). Furthermore, $FXR^{-/-}$ mice develops spontaneously liver tumor with age (Yang et al. 2007). Moreover, Wang et al. (2008) identified FXR as a negative regulator of hepatic inflammation, reporting a reciprocal suppression between FXR and NF-κB signaling pathways. Afterward, Wang and colleagues reported that also the activation of GPBAR1 negatively regulated NF-kB-mediated inflammation and reduced the LPS-induced hepatic inflammation (Wang et al. 2011).

Again, Mencarelli et al. demonstrated that $FXR^{-/-}$ mice show major susceptibility to ConA-induced hepatitis; they found also that the FXR activation with obeticholic acid (OCA) in wild-type mice inhibited the activation of NKT cells, thus reducing the synthesis of osteopontin (OPN) induced by ConA (Mencarelli et al. 2009).

Furthermore, it was shown that the activation of FXR by OCA attenuated liver steatosis, inflammation, and fibrosis in ApoE$^{-/-}$ mice and Zucker rats, two genetic models of NASH (Fiorucci et al. 2004; U.S. Food and Drug Administration (FDA) 2018).

The OCA has been approved for the treatment of the chronic autoimmune liver disease, primary biliary cholangitis (Nevens et al. 2016). Importantly, OCA has shown anti-inflammatory and anti-fibrotic effects in the liver, in the treatment of NASH in a phase II trial (Neuschwander-Tetri et al. 2015) and now is in a phase III clinical trial in NASH patients (Fiorucci et al. 2018b).

In addition to FXR, also the GPBAR1 agonist BAR501 has proved to exert potent anti-inflammatory effects enhancing both energy expenditure in white adipose tissue and vascular endothelial function in mouse models of NASH (Carino et al. 2017a, 2018; Kida et al. 2014).

In a previous study, we have reported the discovery of a non-bile acid dual FXR and GPBAR1 agonist, the 6α-ethyl-3α,7α-dihydroxy-24-nor-5β-cholan-23-ol (christened BAR502) (Festa et al. 2014), that reduced markers of liver inflammation, thus attenuating features of steatohepatitis (Carino et al. 2017b). Recently Högenauer et al. confirmed that other dual FXR and GPBAR1 ligands showed the same effects (Högenauer et al. 2014).

Finally, it was reported by Iracheta-Vellve et al. that both FXR and GPBAR1 ligands inhibited the inflammasome NRLP3 signaling and reduced the expression of IL-1β, thus protecting from liver injury in a mouse model of alcoholic liver disease (ALD) (Iracheta-Vellve et al. 2018).

2.2 Immune-Regulatory Effects of Bile Acids in Myeloid Cells: Monocytes/Macrophages and Dendritic Cells

As previously described, FXR and GPBAR1, the most known bile acid receptors, are expressed by both circulating monocytes and intestinal and liver macrophages (Vavassori et al. 2009; Cipriani et al. 2011; Islam et al. 2011; Maruyama et al. 2002; Mencarelli et al. 2009; Kawamata et al. 2003), although GPBAR1 is the receptor mainly expressed by Kupffer cells, the liver-resident macrophages (Gadaleta et al. 2011). The modulation of monocyte and macrophage activity by BARs was first described for GPBAR1 by Kawamata et al. (2003), and afterward for FXR at both intestinal and hepatic levels (Vavassori et al. 2009; Mencarelli et al. 2009).

It has been widely shown in several studies that the activation of GPBAR1 and FXR in both human and rodent macrophages reduced their pro-inflammatory activity. For instance, Renga et al. reported that FXR modulates the Toll-like receptor 9 (TLR-9) immune-regulatory activity, which elicits the inflammatory responses of intestinal macrophages (Renga et al. 2013). Further, it has been demonstrated that the anti-inflammatory action induced by FXR ligands was mediated by several mechanisms, SHP-dependent and -independent (Vavassori et al. 2009; Renga et al. 2013; Wildenberg and van den Brink 2011).

Indeed, one of these mechanisms through which FXR plays its regulatory activity involves the atypical nuclear receptor small heterodimer partner (SHP), whose expression is generally used to confirm an FXR activation because SHP transcription is directly regulated by FXR (Chanda et al. 2008). SHP acts as a corepressor that promotes the recruitment of other corepressors on the promoters of FXR target genes. Fiorucci et al. in 2004 showed the physical interaction of SHP with c-Jun, the subunit of AP1, thus inhibiting its binding on the promoters of pro-inflammatory genes (Fiorucci et al. 2004). Furthermore, Yang et al. reported that the bond of SHP on the promoter of chemokine CCl2 prevents the recruitment of NF-kB on this promoter (Fig. 2), stabilizing an inhibitor complex that represses the chemokine transcription (Yang et al. 2016).

Additionally, in a recent study by Zhang et al. it has been reported that SHP inhibits the lncRNA H19, and that SHP degradation by Bcl2 increases the expression of H19, thus enhancing the level of pro-inflammatory mediators such as CD31cd1, IL-4, and IL-17 producing CD41 and CD81 cells, in models of cholestasis (Zhang et al. 2016; Song et al. 2017).

Another important mechanism that mediates the immune-regulatory activity of FXR is SHP-independent. When FXR is activated by its ligands, it is recruited on the promoters of several pro-inflammatory genes, including iNOS and IL1-β. This binding stabilizes the complex NCor1 (nuclear receptor corepressor 1) and the

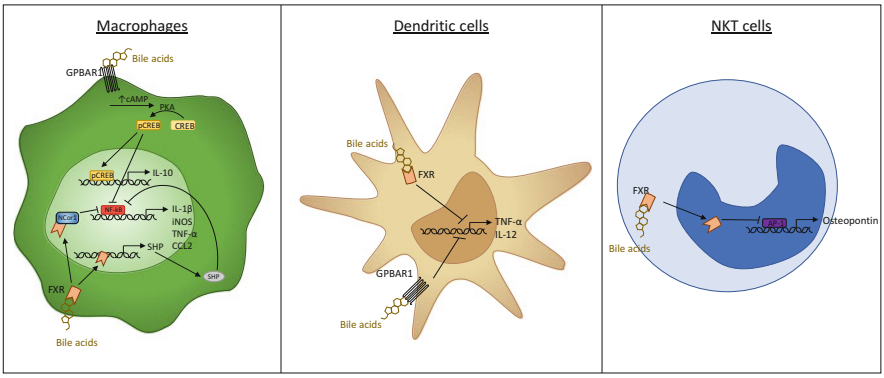

Fig. 2 Bile acid receptors (BARs), G-protein bile acid receptor 1 (GPBAR1), and farnesoid-X-receptor (FXR) are expressed in cells of innate immunity and their activation exerts anti-inflammatory effects. Macrophages and dendritic cells (DCs) express both GPBAR1 and FXR receptors, while for NKT cells there is currently only evidence of the expression of FXR. In macrophages, the anti-inflammatory action induced by FXR ligands was mediated by several mechanisms, SHP-dependent, which inhibit the recruitment of NF-kB on the promoters of pro-inflammatory genes, and SHP-independent, which stabilize the binding of NCor1 complex on the promoters of pro-inflammatory genes. The activation GPBAR1 directly activated the IL-10 transcription in a promoter-specific manner, by a GPBAR1-PKA-CREB pathway that results in a GPBAR1-dependent recruitment of CREB to a specific responsive element CRE on the IL-10 promoter, inducing a polarization toward the anti-inflammatory M2 macrophage phenotype. In DCs cells, both FXR and GPBAR1 ligands downregulate the production of TNF-α and IL-12. In NKT cells, bile acids reduced the expression of osteopontin via FXR/SHP-c-Jun axis

NCor1-containing complexes linked to NF-kB-response element, which are normally linked on the promoters of these genes in basal conditions, thus preventing the direct engagement of kB subunit (Cipriani et al. 2011; Islam et al. 2011). Conversely, the activation of TLR-4 causes the release of NCor1 from the promoters allowing the transcriptional activation of these genes (Vavassori et al. 2009). It has been confirmed, through silencing experiments using an anti-NCor1 siRNA, that the OCA, also known as INT-747, induces an activation of FXR that results in a repression of iNOS and IL-1β transcription, due to the stabilization of NCor1 on their promoters (Fig. 2) (Cipriani et al. 2011; Mencarelli et al. 2009). The regulatory effects of FXR that involves NF-κB, AP1, and NCor1 could explain some of the beneficial effects exerted by FXR agonists. As reported in several studies, $Fxr^{-/-}$ mice present an impaired expression of inflammatory mediators and increased intestinal permeability with age (Cipriani et al. 2011), and when exposed to DSS or TNBS, they develop a severe disease (Cipriani et al. 2011; Maruyama et al. 2002). Furthermore, in human samples of Crohn's disease and ulcerative colitis, as well as in FXR wild-type mice treated with TNBS or DSS, a severe intestinal inflammation was found, as confirmed by higher amounts of NF-κB-dependent cytokines, including TNF-α, IL-6, IL-1β, and INF-γ, associated with a reduced expression of FXR (Vavassori et al. 2009). It has been shown that treating wild-type mice exposed to DSS or TNBS with OCA results in a reduced expression of pro-inflammatory marker genes such as IL-1β, IL-6, and monocyte chemoattractant protein 1, thus confirming the immune-modulatory activity of FXR, which involves both NF-KB-dependent and -independent pathways (Vavassori et al. 2009; Gadaleta et al. 2011; Kida et al. 2014; Massafra et al. 2016).

Furthermore, the GPBAR1 ligands, including the endogenous bile acids DCA and LCA (Maruyama et al. 2002; Kawamata et al. 2003), or other synthetic agonists such as INT-777 and BAR501, regulate the TLR-4-induced activation of spleen and intestinal macrophages. It has been shown that the GPBAR1 signaling involves the activation of PKA (protein kinase A) via cAMP, and the recruitment of CREB (cAMP-responsive element-binding protein) on the promoters of specific target genes (Robinson et al. 2016; Schubert et al. 2017; Swann et al. 2011; Keane et al. 1984). A study by Haselow et al. demonstrated that this pathway cAMP-PKA-CREB reduces the activity of NF-kB by a STAT1-dependent mechanism (Fig. 2) (Haselow et al. 2013). Recently our group reported that the BAR501, a selective agonist of GPBAR1, rescues mice from colitis by regulating the activation of intestinal macrophages (Biagioli et al. 2017). In particular, the GPBAR1 activation leads to a shift of the polarization of colonic macrophages from a M1 (CD11b + Ly6C− CCR7+ CD38+ IL-6+) phenotype to a M2 anti-inflammatory (CD11b + Ly6C− CCR7− Egr2+ IL-10+) phenotype. Conversely, the $GPBAR1^{-/-}$ mice are characterized by a prevalent M1 macrophage population, thus developing a severe colitis in response to TNBS. Furthermore, it has been reported that the GPBAR1 agonism reverses the TNBS effects, reducing intestinal and circulating monocytes/macrophages, but does not modifies the ratio of resident (non-classical monocytes CD11b + CCR2− CX3CR1hi Ly6Clow CD11c+) versus inflammatory monocytes (classical monocytes, CD11b + CCR2+ CX3CR1low Ly6Chi CD11c−) (Biagioli

et al. 2017). These results confirm that the differentiation of monocytes toward a pro- or anti-inflammatory phenotype is not dependent only on the Ly6C expression, and that their differentiation within the tissues is driven by the organ microenvironment (Zundler and Neurath 2017).

Moreover, through ex vivo studies in human and murine macrophages, Yoneno et al. confirmed that the exposure of these cells to GPBAR1 agonists results in a direct reduction of the pro-inflammatory cytokines expression, such as IFN-γ, IL-1β, IL-6, and TNF-α, along with an increase of the expression of anti-inflammatory cytokines, particularly IL-10 (Yoneno et al. 2013). It was further suggested that the beneficial effects exerted by GPBAR1 agonists were strongly associated with increased transcription of IL-10 in the intestine, and conversely they were lost in IL-$10^{-/-}$ mice (Biagioli et al. 2017). Finally, it was shown using human and murine macrophages that the activation of GPBAR1 directly activated the IL-10 transcription in a promoter-specific manner, by a GPBAR1-PKA-CREB pathway that results in a GPBAR1-dependent recruitment of CREB to a specific responsive element CRE on the IL-10 promoter (Fig. 2) (Biagioli et al. 2017).

In addition, it has been reported that the GPBAR1 activation by NF-kB-independent mechanisms reduces the accumulation of activated macrophages in aortic plaques and adipose tissues (Pols et al. 2011; Perino et al. 2014). By using genetic models, i.e., the Gpbar1-deficient bone marrow chimeric mice and the myeloid cell-specific Gpbar1-knockout mice, they have shown that the selective ablation of GPBAR1 causes an impaired activation of a multi-protein aggregate formed by AKT–mTOR complex 1 (AKT–mTORC1), and the translation of the liver inhibitory protein isoform of the transcription factor CCAAT/enhancer binding protein β, resulting in the development of a pro-inflammatory phenotype (Perino et al. 2014). Furthermore, the exposure of these selective Gpbar1-deficient mice to a high-fat diet worsens the insulin resistance and inflammation in adipose tissues.

These results confirmed the involvement of FXR and GPBAR1 in the regulation of monocytes and macrophages, and highlight the potential therapeutic use of their ligands in the treatment of intestinal and hepatic inflammatory diseases.

Interestingly McMahan et al. described that the treatment of obese db/db mice, a genetic model of NAFLD, with INT-767, a dual FXR/GPBAR1 agonist, rescues from liver injury, improving liver histology, increases the M2 macrophage markers (CD206, Retnl and Clec7a) and the proportion of intrahepatic monocytes Ly6C low, with an anti-inflammatory phenotype. In addition, they confirmed these data in vitro, showing that treating monocytes with INT-767 decreased Ly6C expression and increased IL-10 production via cAMP (McMahan et al. 2013). Furthermore, our group has reported that treating mice exposed to a high-fat diet (HFD), with BAR501, a GPBAR1 selective ligand, or BAR502, a dual FXR/GPBAR1 ligand, ameliorated the steatosis and fibrosis scores, thus attenuating the fat liver deposition, reduced the expression of pro-inflammatory markers and induced a shift of macrophage polarization to M1 phenotype toward a M2 phenotype (Carino et al. 2017b; Ramírez-Pérez et al. 2017). Högenauer et al. recently confirmed the same results using other dual FXR/GPBAR1 ligands (Högenauer et al. 2014).

In addition to monocytes/macrophages, expression of GPBAR1 and FXR has been detected in dendritic cells (DCs). DCs are essential sentinels of the intestinal epithelium where they are able to sense pathogens and direct the appropriate immune response to maintain tissue homeostasis, acquiring a functional phenotype able to increase or attenuate inflammatory responses (Gadaleta et al. 2011; Massafra et al. 2016; Ichikawa et al. 2012). Gadaleta et al. (2011) demonstrated that the activation of FXR by a selective agonist (INT-747) reduces the severity of colitis induced in mice by both DSS and TNBS, decreasing the production of pro-inflammatory cytokine TNF-α in DCs (Fig. 2). Furthermore, the activation of FXR attenuates the differentiation of circulating monocytes in DCs. The beneficial effect of FXR activation on DSS-induced colitis was also confirmed by Massafra et al. (2016). They demonstrated that the activation of FXR by OCA promotes an anti-inflammatory state with an increase in the retention of DCs in the spleen, which leads to a decrease of these cells in the colon, inducing a decrease in colon inflammation. Interestingly, FXR activation modulates the chemotactic environment in the colonic site of inflammation, as Madcam1 expression is decreased, while Ccl25 is upregulated. Ichikawa et al. instead investigated the role of GPBAR1 in DCs (Ichikawa et al. 2012). Their studies demonstrated that the activation of GPBAR1 in DCs induces a lower production of IL-12 and TNF-α in response to bacterial antigens (Fig. 2). Moreover, the differentiation of DCs in the presence of Gpbar1 agonists induces an IL-12 hypo-producing phenotype.

2.3 Immune-Regulatory Effects of Bile Acids in Lymphoid Cells

While the action of bile acids mediated by the activation of BARs in myeloid cells has been studied for a long time and is now well characterized, only little is known about the role of bile salts in lymphoid cells like NK, NKT, CD4+ or CD8+ T cells, and B cells (Vavassori et al. 2009; Maruyama et al. 2002). T and B lymphocytes express low levels of FXR and GPBAR1, and it is unclear whether that results in any functional activity. In contrast, the activation of these receptors on NKT cells results in several regulatory functions that are inhibitory in nature. In recent years, studies have focused on NKT cells and on the action of the FXR receptor in the liver.

In 2009, Mencarelli et al. showed that, in the liver, the NKT cells express a functional FXR whose activation decreases the inflammation promoted by these cells (Mencarelli et al. 2009). They found that FXR gene ablation results in a time-dependent increase of liver expression (up to 20-fold in a 9-month-old mouse) of IL-4, IFN-γ, and osteopontin; in addition, contrast to wild-type mice, $FXR^{-/-}$ mice administered with concanavalin A (Con A) develop a lethal hepatitis characterized by a robust increase of pro-inflammatory mediators, supporting the regulatory role of FXR in NKT cells. The data obtained showed that the activation of FXR in NKT cells reduces the expression of pro-inflammatory cytokines such as IL-1β, TNF-α, and IFN-γ both in vitro and in vivo models of ConA-induced hepatitis. The protective role in the model of autoimmune hepatitis induced by ConA is associated with a lower influx of NKT cells in the liver and less activation of these cells, as shown by

the downregulation of osteopontin expression (Fig. 2). Finally, Mencarelli et al. demonstrated that the downregulation of osteopontin is mediated by the axis FXR/SHP-c-Jun (Mencarelli et al. 2009).

In another study published in Science in 2018, Ma et al. studied the interaction between intestinal microbiota, bile acids, NKT cells, and liver cancer (Ma et al. 2018). For this study, they used one primary liver model of tumor and three liver metastasis models. They demonstrated that alterations in the microbiota composition change the bile acid pool by modifying the immune response to hepatic tumors. In this study, they state that secondary bile acids (known ligands of GPBAR1) decrease the accumulation of NKT cells in the liver, thus attenuating the response of the immune system against the tumor. In contrast, Ma et al. claimed that primary bile acids, such as CDCA, increase the accumulation of NKT cells in the liver by increasing the expression of chemokine CXCL16 on the hepatic sinusoids, thus favoring the immune response against the tumor. Therefore, Ma et al. reported that the primary bile acids, which are known FXR ligands, promote an enhanced immune response, in contrast to what was observed in the previous studies (Mencarelli et al. 2009).

There are no studies currently available in the literature regarding the action of FXR and GPBAR1 on NKT cells in organs other than the liver or on the effect of these receptors on other lymphoid cells. Further studies are needed to determine whether FXR and GPBAR1 might exert immune-modulatory effects in liver diseases.

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Structural Insight into the Binding Mode of FXR and GPBAR1 Modulators

Francesco Saverio Di Leva, Daniele Di Marino, and Vittorio Limongelli

Contents

Abstract

In this chapter we provide an exhaustive overview of the binding modes of bile acid (BA) and non-BA ligands to the nuclear farnesoid X receptor (FXR) and the G-protein bile acid receptor 1 (GPBAR1). These two receptors play a key role in

F. S. Di Leva
Department of Pharmacy, University of Naples "Federico II", Naples, Italy

D. Di Marino
Faculty of Biomedical Sciences, Institute of Computational Science, Center for Computational Medicine in Cardiology, Università della Svizzera italiana (USI), Lugano, Switzerland

Department of Life and Environmental Sciences, Polytechnic University of Marche, Ancona, Italy

V. Limongelli (✉)
Department of Pharmacy, University of Naples "Federico II", Naples, Italy

Faculty of Biomedical Sciences, Institute of Computational Science, Center for Computational Medicine in Cardiology, Università della Svizzera italiana (USI), Lugano, Switzerland

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many diseases related to lipid and glucose disorders, thus representing promising pharmacological targets. We pay particular attention to the chemical and structural features of the ligand-receptor interaction, providing guidelines to achieve ligands endowed with selective or dual activity towards the receptor and paving the way to future drug design studies.

Keywords
Bile acids · FXR · GPBAR1 · Homology modelling · Molecular docking · Molecular dynamics (MD) · X-ray crystallography

1 Introduction

Since the advent of computer simulations in chemistry in the 1960s, it was clear that the computational methods would have impacted on science through simulations able to predict the molecular processes that occur at cellular level. The recent and continuous growth of the computing power has further increased the expectations towards in silico studies, which have indeed played in the recent years an even more central role especially in drug design (Schneider 2018). Among the most successful techniques is molecular docking that allows elucidating the physico-chemical properties underlying the binding interaction between a ligand and its molecular target. In order to perform a docking calculation, the user defines a region in the target structure that includes the binding site, and the docking algorithm samples all the possible modes of interaction between the ligand and the protein within that region. At the end of the calculation, the docking program ranks all the ligand binding conformations using a scoring function, with the best ligand binding modes listed as the top scored poses. The very strength of docking is the speed of the calculation that allows the evaluation of even thousand compounds with an affordable computational cost (i.e., virtual screening). The weakness is the inaccuracy of the sampling that is reflected in scoring functions poorly correlated with the experimental ligand binding constants, when available, and the weak prediction of the ligand binding mode (Kontoyianni et al. 2004). However, docking has signed an époque in which medicinal chemists have successfully combined docking calculations and experimental data to disclose the binding mode of many drugs and in turn explaining their mechanism of action. Furthermore, the elucidation of the intermolecular forces established by the ligand with its target, like salt bridges, H-bonds and hydrophobic contacts, paves the way to rational modifications of its structure to achieve compounds with improved potency. This poses docking calculations in a privileged position in structure-activity relationship (SAR) studies and structure-based drug design.

Despite its wide use, docking calculations suffer of many limitations. Among the most severe is the lack of dynamic models for protein flexibility and solvent molecules. Although many efforts have been paid to overcome such limitations, it seems that docking evolution has reached a plateau and a breakthrough is awaited in the field. In this framework, molecular dynamics (MD) represents a valuable tool in

support of docking. During the MD calculation, the forces operating on each atom of the system and their derivatives are computed, and the motion of the atoms obeys to Newton's equation of motion generating a time-dependent trajectory. In such a way, it is possible to study the event under investigation in the real-time scale. Furthermore, in MD simulations the ligand, the protein and the solvent molecules are typically explicit and fully flexible, thus overcoming the main limitation of the docking calculations. Unfortunately, the application of MD in drug design is limited by the long time scale of the ligand/protein binding event, which ranges from microsecond to seconds. As a result, MD calculations can only be used to describe partially the ligand binding event and risk to miss important parts of the ligand/protein interaction. As a result, the most common application of MD in medicinal chemistry is to validate the docking poses and to provide more accurate data on the ligand/protein binding interaction. It can be foreseen that the situation might significantly improve in the near future, thanks to the development of even more powerful computing resources such as superfast computers, posing MD calculations in a leading role in future drug design studies (Copeland 2016). The MD simulations can be also used to perform more rigorous calculations of the protein/ligand binding affinity through free energy techniques. Among the most widely used methods are Molecular Mechanics Poisson-Boltzmann Surface Area, Free Energy Perturbation, Thermodynamic Integration and Funnel-Metadynamics (Gilson and Zhou 2007; Limongelli et al. 2013). Despite the accuracy of such methods, their massive application in drug design is hampered by their larger computational cost if compared to docking calculations, which however pay in terms of results' accuracy as previously explained. Nevertheless, the improvement of the MD simulation codes and the exponential increase of the computing power make possible to perform these calculations on dozens of compounds, and their impact on future drug design studies is easily foreseeable.

Both docking and MD calculations have been used to study the binding interaction of ligands to the bile acid receptors. In this chapter, we limit our discussion to the description of the binding mode of ligands to the bile acid receptors FXR and GPBAR1 reported in literature (Figs. 1 and 2). From this amount of data, we depict the molecular requisites for a ligand to operate as either selective binder or dual FXR and GPBAR1 ligand. As conclusion, a perspective on computer-aided drug design on these two targets is provided.

2 Structure of the Bile Acid Receptors

Having the 3D structure of the molecular target is the *conditio sine qua non* to perform successful structure-based drug design studies. While the FXR receptor has been resolved by X-ray in complex with different ligands, an experimental GPBAR1 structure is not available so far. However, using modern computational techniques, in particular homology modelling, different research groups have built a 3D model of the receptor and validated their results through a series of in silico and experimental data. A detail description of the ligand binding site of FXR and GPBAR1 is reported

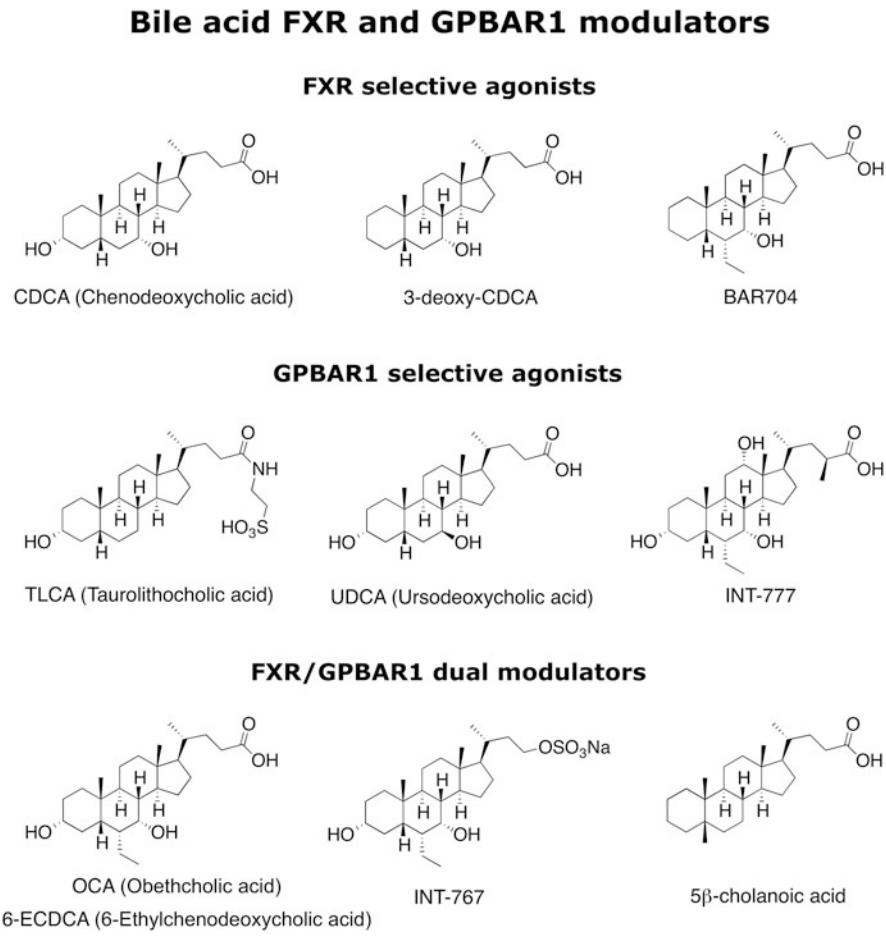

Fig. 1 Principal endogenous and synthetic BA derivatives reported in the chapter as FXR and GPBAR1 modulators

in the following paragraphs. We note that in the whole chapter, we use the amino acid numbering of the human sequence for either the receptor.

2.1 The Farnesoid X Receptor (FXR) Structure

The farnesoid X receptor (FXR) is a member of the nuclear receptors (NRs) superfamily and is composed of two principal structural domains: (1) the central DNA-binding domain, which allows the receptor to recognize specific DNA sequences in the nucleus, and (2) the ligand-binding domain (LBD) where endogenous and exogenous molecules bind modulating the receptor activity. In particular, the ligand binding to the LBD triggers receptor conformational changes that favour

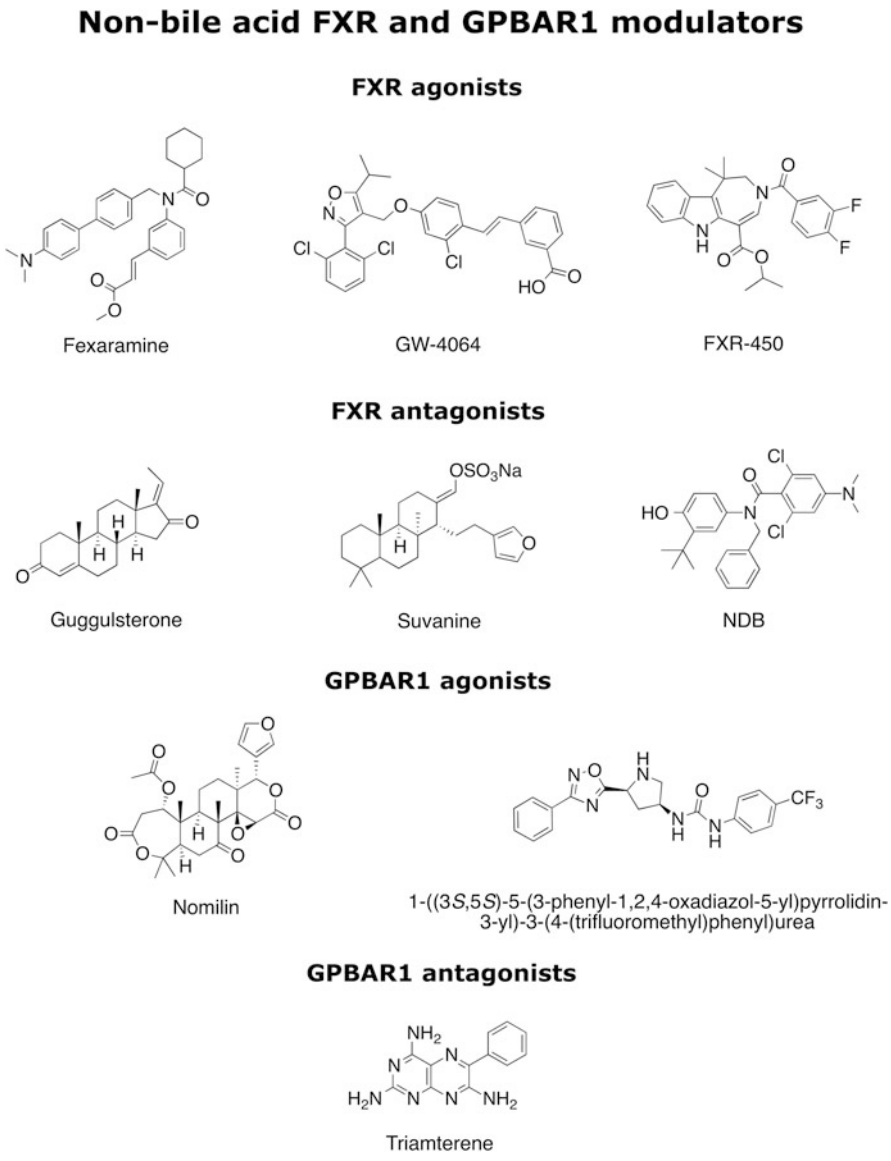

Fig. 2 Principal non-BA compounds reported in the chapter as FXR and GPBAR1 modulators

the release of corepressors and the recruitment of coactivators, which in turn results in the transcription of the target genes (Downes et al. 2003). The FXR-LBD (amino acids 248–472) is composed exclusively of α-helices, and the clear topological representation of its secondary structure elements is reported in Fig. 1a. The first tridimensional (3D) structure of the FXR-LBD was resolved in 2003 by X-ray

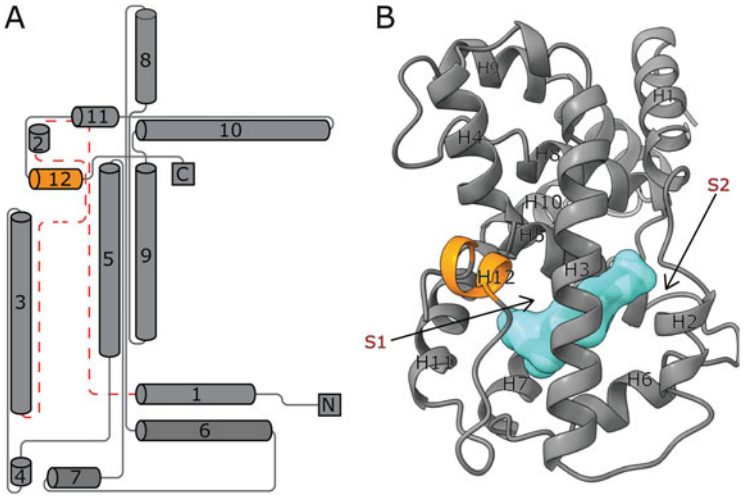

Fig. 3 (**a**) Topological representation of the FXR-LBD. The activation function-2 (AF-2) domain represented by H12 is highlighted in orange, and the unstructured region between H1 and H3 is represented as a dashed red line. (**b**) Ribbon representation of the FXR-LBD. The H12 is coloured in orange and the ligand molecular surface into the binding site is shown in cyan. S1 and S2 represent the primary (orthosteric) and the secondary (allosteric) ligand binding sites, respectively

crystallography (Downes et al. 2003; Mi et al. 2003). Later on, a number of crystal structure of the FXR-LBD have been reported (Akwabi-Ameyaw et al. 2008; Soisson et al. 2008; Flatt et al. 2009; Jin et al. 2013; Lu et al. 2018). These structures show that the FXR-LDB adopts a 12 α-helices bundle (Fig. 3), similar to other NRs such as RXRα, PXR, VDR, PPARs and RORβ. At variance with the other NRs, however, FXR shows a more extended helix (H) 6, which replaces the β-turn following H5, and a disordered region between H1 and H3 instead of H2 (Fig. 3a). These elements play an active role during the switch from the inactive to the active state of the receptor. Remarkably, the activation function-2 (AF-2) domain of the FXR-LBD, namely, H12, is close to H3 and H4 (Fig. 3b) forming a hydrophobic cleft to which the coactivator peptides bind through the lipophilic interface of their LXXLL helical motif (Downes et al. 2003; Mi et al. 2003).

2.2 The Cell-Surface G-Protein-Coupled Bile Acid Receptor 1 (GPBAR1) Structure

The cell-surface G-protein-coupled bile acid receptor 1 (GPBAR1, TGR5, M-BAR) belongs to the class A (i.e. rhodopsin-like superfamily) G-protein-coupled receptors (GPCRs). The bile acids (BAs) are the endogenous ligands of GPBAR1 that modulates specific non-genomic signalling pathways. The activation of this receptor results in the increase of intracellular cAMP levels, which in turn activates specific intracellular signalling cascades. This receptor plays a key role in different organs,

such as liver, intestine, kidneys, spleen, heart and lungs (Tiwari and Maiti 2009). In humans, a single exon located on chromosome 2 encodes for the 330 amino acid sequence of GPBAR1 (Tiwari and Maiti 2009). The receptor protein sequence is well-conserved among different organisms (Kawamata et al. 2003), also sharing a modest sequence similarity with the receptor family of the endothelial differentiation gene (EDG) (Tiwari and Maiti 2009).

Unfortunately, the three-dimensional (3D) structure of GPBAR1 is not known; however the receptor shares the structural features common to all GPCRs. In details, GPBAR1 is composed of seven transmembrane (TM) α-helices that are connected by alternating intracellular and extracellular loops, ICL and ECL, respectively. The N-terminus is pointed towards the extracellular side, whereas the C-terminus points towards the intracellular side (Mustafi and Palczewski 2009) (Fig. 4a). GPCRs can be grouped in six classes that are further divided in subgroups based on sequence homology and functional similarity (Mustafi and Palczewski 2009). Members of different subgroups, playing similar or specific functions, can however show high degree of sequence similarity. The homology between sequences is classified based on the presence of similar amino acids in specific positions, the structural motifs and the nature of the ligands (Fig. 4a).

In all the GPCRs, including GPBAR1, the binding of an agonist or an antagonist molecule produces a structural reorganization that stabilizes the receptor in the active (agonist bound) or inactive (antagonist bound) state, respectively. Knowing the three-dimensional (3D) structure of GPBAR1 is therefore essential to perform structure-based drug design studies aimed at developing novel agonist and antagonist molecules. In the recent years, a number of GPBAR1 structural models have been proposed through the application of the homology modelling technique employing as template the X-ray structure of different GPCRs (Fig. 4a). This condition led to build receptor models differing in some features such as (1) the length of the transmembrane α-helices; (2) the length of the intracellular and extracellular loops; and (3) the orientation of the side chains of the amino acids that compose the predicted GPBAR1 binding pocket. As a consequence, even the binding modes of both natural (e.g. CA, CDCA, LCA, TLCA, DCA, linoleic acid, OA, obacumone) and synthetic (e.g. INT-777, TRC210258, WB403) ligands elucidated using such models can be different (Guo et al. 2016). The GPBAR1 model was obtained using one single template such as (1) adenosine receptor A_{2A} (Fig. 4b); (2) rhodopsin; (3) sphingosine-1-phosphate receptor 1 (S1P receptor 1); and (4) β2-adrenergic receptor and also using (5) multiple templates that include parts of different receptors like β2-adrenergic receptor, adenosine receptor A_{2A}, sphingosine-1-phosphate receptor 1, dopamine receptor D_3, muscarinic receptor M_2 and chemokines receptor CXCR4. In general the GPBAR1 sequence identity and similarity of the TM α-helices with the different templates range between 18–20% and 40–55%, respectively (Gertzen et al. 2015), suggesting that all the models generated so far can be possible good candidates for representing the "real" GPBAR1 structure. Furthermore, many of the GPBAR1 models were validated both experimentally and computationally through mutagenesis, MD and rational drug design studies (Gioiello et al. 2012; Macchiarulo et al. 2013; D'Amore et al. 2014;

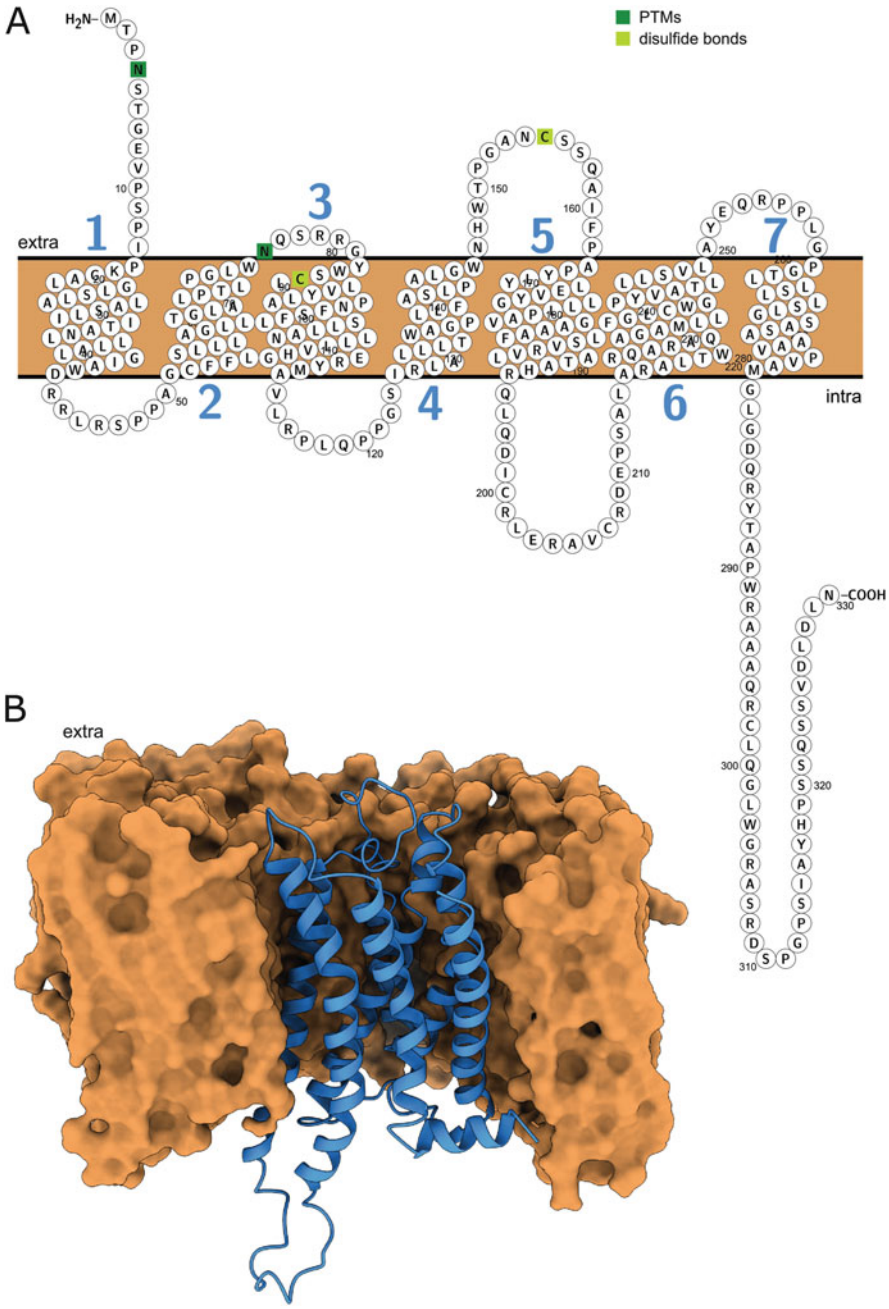

Fig. 4 (**a**) Topological representation of GPBAR1. The sequence and the organization of the 7 transmembrane (TM) helices are reported. The phospholipid bilayer is coloured in light brown. The residues subjected to posttranscriptional modifications (PTMs) and the cysteines involved in disulphide bridges are also highlighted. (**b**) Ribbon representation of the 3D model of GPBAR1 embedded in the phospholipid bilayer. The model was provided by D'Amore et al. (2014). The GPBAR1 is coloured in light blue and the membrane is represented as light brown surface

Sepe et al. 2014; Di Leva et al. 2015; Gertzen et al. 2015; Sindhu and Srinivasan 2015a, b; Yu et al. 2015). In Fig. 2b the 3D model of the GPBAR1 receptor developed by D'Amore et al. is reported embedded in a 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) phospholipid bilayer (D'Amore et al. 2014). This model has been successfully used in different drug design studies to develop selective GPBAR1 agonist molecules (D'Amore et al. 2014).

Despite the different procedures used to build the GPBAR1 3D structure, there are some sequence/structural characteristics that are shared among all the models. In detail, the disulphide bridge formed by the residues Cys73 and Cys143 and the position of the residues Glu97, Arg98 and Tyr99 on TM3 (Fig. 4a, b) are preserved as these residues are highly conserved among the different GPCRs used as template (D'Amore et al. 2014; Gertzen et al. 2015). It is important to underline that the residues forming the GPBAR1 binding site, which were identified through molecular modelling and mutational analyses, are almost the same in all the models. Therefore, for the sake of drug design, the reliability of the different receptor models is not questionable, while the elucidation of the binding mode of the endogenous and synthetic ligands in the GPBAR1 binding site remains a challenging task. A recent interesting study by Spomer et al. elucidated the structure and the function of the C-terminal portion of the receptor by using MD simulations and experimental protocols (Spomer et al. 2014). In particular, the authors found a direct correlation between the receptor localization at the level of the plasma membrane and its activation by ligands through the presence of a long α-helix ($\geq$9 residues) located at the C-terminus of the GPBAR1. Finally, Greife et al. have reported in a recent work the ability of GPBAR1 to form homodimers and higher-order oligomers (Greife et al. 2016). These authors have employed FRET experiments, multiparameter fluorescence image spectroscopy (MFIS) and molecular modelling to characterize the structural organization of the GPBAR1 oligomers, also suggesting which TM α-helices are directly involved in the stabilization of the oligomers interfaces.

In conclusion, the lack of the X-ray structure of GPBAR1 represents a limitation for structure-function relationship studies. However, computational techniques, such as homology modelling, molecular docking and MD simulations, provide reliable models of binding of BAs to GPBAR1 that are able to support pharmacological and drug design studies as described in detail in the following paragraphs.

3 Exploiting the Bile Acid Scaffold to Achieve FXR and GPBAR1 Modulation

The endogenous ligands of FXR and GPBAR1 are BAs that are the product of the cholesterol degradation. BAs are involved in the promotion and regulation of several cellular processes, and for this reason they have acquired an increasingly remarkable relevance in pharmacology as promising scaffold to develop novel modulators of BAs receptors. In this scenario, many research groups have modified the chemical structure of endogenous BAs to achieve new ligands endowed with selective or dual activity towards FXR and GPBAR1. In the following paragraphs, we provide a

detailed description of the binding modes of the most potent BA molecules active as agonists and antagonists of FXR and GPBAR1. The chemical and structural features of the ligand-receptor interaction are most useful for future drug design applications.

3.1 FXR Agonists

The ligand binding site of FXR is formed by helices 3, 5, 7, 10, 11 and 12, which define a large amphipathic pocket that can host both endogenous BAs and BA derivatives, as well as synthetic organic compounds (Mi et al. 2003; Akwabi-Ameyaw et al. 2008). In this paragraph we focus on the ability of the FXR-LBD to recognize BA derivatives based on their peculiar shape and chemical features. From the structural point of view, the steroidal nucleus of BAs comprises a *cis* A/B ring juncture which provides a tilted molecular surface with two distinct faces: (1) a β-convex hydrophobic face and (2) a α-concave hydrophilic face on which hydroxyl groups can be found at the 3, 7 and 12 positions. The number and spatial configuration of the latter groups can vary among the different BAs, modulating their affinity and selectivity profile. Moreover, endogenous BAs are endowed with a five- to eight-atom side chain ending with a negatively charged group such as a carboxylic or sulfonic acid, which also influences their pharmacodynamic properties (Hofmann et al. 2010).

Among the endogenous BAs, chenodeoxycholic acid (CDCA) is the most potent ligand of FXR (Makishima et al. 1999; Parks et al. 1999). Over the last decades, numerous semi-synthetic analogues of CDCA have been developed as agonists of this receptor (Sepe et al. 2015; Massafra et al. 2018). Among these is obeticholic acid (OCA), also known as 6α-ethyl-chenodeoxycholic acid (6-ECDCA) (Pellicciari et al. 2002), which has been recently approved by FDA for the treatment of primary biliary cholangitis. In 2001, the resolution of the crystal structure of FXR from *Rattus norvegicus* (rFXR) in complex with OCA and another FXR agonist, namely 3-deoxy-CDCA, disclosed for the first time the molecular basis of bile acid/FXR interaction (Mi et al. 2003). Remarkably, rFXR-LBD shares the 95% of homology with the human FXR-LBD (hFXR-LBD), with all of the residues in the ligand binding site conserved among the two species. This observation suggested that BAs could establish similar interaction patterns in the binding site of both rFXR and hFXR. In detail, both OCA and 3-deoxy-CDCA bind to the FXR-LBD orienting their A ring towards H11 and H12 and D ring towards H3 and H5 (Fig. 5a). In this pose, the ligand steroidal skeleton establishes several van der Waals interactions in the FXR-LBD, contacting the side chain of residues such as Met290 and Ala291 on H3, Met328 on H5 and Ile362, Phe366 and Tyr369 on H7. Further hydrophobic contacts are formed by the 6α-ethyl of OCA, which increases the receptor affinity and the potency of this compound. Notably, the carboxylate group of both ligands forms a salt bridge with the side chain of Arg331, which represents the major driving force for orienting BAs in the FXR-LBD. Indeed, semi-synthetic steroidal ligands in which the 3-OH is replaced by a carboxylate group assume an inverted binding mode

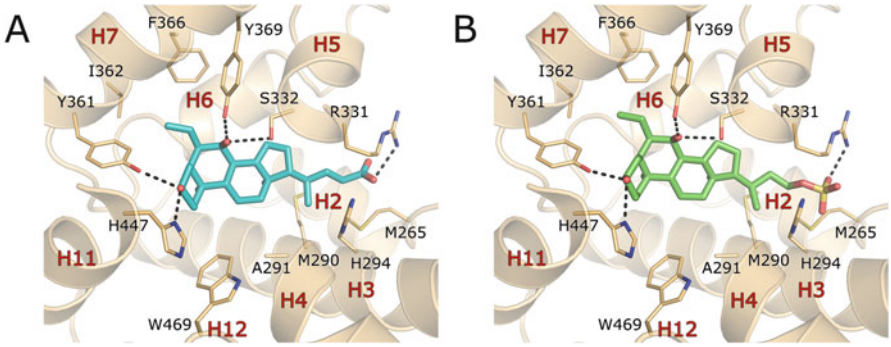

Fig. 5 The binding mode of (**a**) OCA (cyan sticks) (Mi et al. 2003) and (**b**) INT-767 (green sticks) (D'Amore et al. 2014) to the FXR-LDB. The receptor is shown as orange cartoon, with the ligand interacting amino acids represented as orange sticks. H8 and H10 and nonpolar hydrogens are not displayed for clarity. Hydrogen bonds are shown as dashed black lines

with the A ring oriented towards H3 and H5 and the D ring facing H12 (Soisson et al. 2008). On the other side of the binding cavity, the 7α-OH of OCA establishes H-bonds with the side chains of Ser332 and Tyr369. Furthermore, the ligand forms a H-bond with the protonated His447 on H11 through its 3α-OH group (Fig. 5a). This contact stabilizes the cation-π interaction between the side chains of His447 on H11 and Trp469 on H12, which functions as a receptor activation trigger stabilizing H12 in a conformation competent for the recruitment of the coactivator peptides (Mi et al. 2003). This mechanism is peculiar of FXR and few other nuclear receptors such as LXRα and LXRβ (Williams et al. 2003). Differently from OCA, 3-deoxy-CDCA, which is deprived of the 3α-hydroxy group, cannot form the H-bond with His447. Nevertheless, its A ring can stabilize the cation-π interaction between His447 and Trp469 through an extensive network of hydrophobic contacts, however, inducing the activation of the receptor (Mi et al. 2003). This indicates that the 3α-OH, which is the most recurring polar function in bile acids, is not essential to achieve FXR agonism. In addition, the lack of the 3α-OH group can increase the ligand selectivity against other BAs receptors like GPBAR1, in which it is fundamental for the receptor activation. For instance, BAR704, namely, the 3-deoxy derivative of OCA, is a potent and selective FXR agonist (Carino et al. 2018). In order to gain selectivity over GPBAR1, the 3α-OH was alternatively replaced by other functional groups. An example is the 3β-azido analogue of OCA that is a potent FXR ligand devoid of any activity towards GPBAR1. Its activity profile can be explained by the dipole moment of the azido group that induces a negative charge on the distal nitrogen atom at C-3, which stabilizes the interaction with the positively charged His447 in FXR, while disfavours that with the negatively charged amino acid Glu169 in GPBAR1 (see Sect. 3.3) (Festa et al. 2017). At variance with the 3α-OH group, either the removal of the 7α-OH or the inversion of the configuration at C-7 fully abolishes the ligand binding affinity to FXR. In fact, the 7β-epimer of CDCA, ursodeoxycholic acid (UDCA), and its derivatives are inactive towards the

receptor (Sepe et al. 2014). This reveals that both the presence and orientation of the 7α-OH are crucial to form the H-bonds with Ser332 and Tyr369 and that these are essential for FXR agonism. Furthermore, a more recent work has demonstrated that the introduction of additional hydroxyl groups on the BAs scaffold, specifically the insertion of the 11β-OH group, increases the FXR selectivity over GPBAR1. In fact, computational studies on the 11β-OH analogue of OCA have shown that a β-hydroxyl moiety on the carbon 11 of the steroidal scaffold can establish an additional H-bond in the FXR-LBD (Pellicciari et al. 2016), while in GPBAR1 it may point to a hydrophobic region where no favourable interactions are possible.

The FXR agonist properties and selectivity profile of BAs can be further modulated by varying the length and the nature of their side chain. Here, the replacement of the carboxylate group with other polar functions such as an alcohol or a sulphate group is generally well tolerated in FXR agonists (Rizzo et al. 2010; D'Amore et al. 2014; Festa et al. 2014). In fact, these groups are all able to interact, similarly to carboxylate, with the side chain of Arg331 (Fig. 5b), which is known to play a key role in the receptor activation mechanism (D'Amore et al. 2014). This interaction is particularly favoured in the case of linear side chains featuring two or three methylene units (C23 or C24 bile acids), which also slightly improves the FXR/GPBAR1 selectivity ratio (Festa et al. 2014). On the other hand, BAs presenting elongated and branched side chains, show weaker FXR agonist properties while they are endowed with good affinity towards GPBAR1 (Pellicciari et al. 2009; Gioiello et al. 2011). It is worth noting that compounds with a long hydrophobic side chain were found to occupy a noncanonical binding pocket of the FXR-LBD, known as S2 or 'back door', shaped by the loop connecting H1 and H2. Computational and experimental studies suggest that the occupancy of this site can influence the position of H3 and H12 and in turn the activation state of the receptor. At this regard, Gioiello et al. reported a set of CDCA derivatives with a long carbamate side chain that show FXR activity profiles ranging from full to partial agonism and antagonism. The authors ascribe the diverse activity profiles to the different interactions established by the ligands with the S2 site and the ligand-induced fit effects on the neighbouring helices such as H3 involved in the receptor activation (Massafra et al. 2018).

3.2 GPBAR1 Agonists and Antagonists

In the last years, several efforts have been dedicated to the discovery and development of potent and selective GPBAR1 agonist molecules. The majority of the ligands studied so far are BAs; however a small group of non-bile acid (non-BA) molecules were also characterized. In order to identify the binding mode of different BAs and synthetic ligands, the combination of diverse approaches, including computational chemistry, biochemistry and cellular and molecular biology, has been used (Macchiarulo et al. 2013; D'Amore et al. 2014; Duboc et al. 2014; Sepe et al. 2014; Di Leva et al. 2015; Fiorucci and Distrutti 2015; Gertzen et al. 2015).

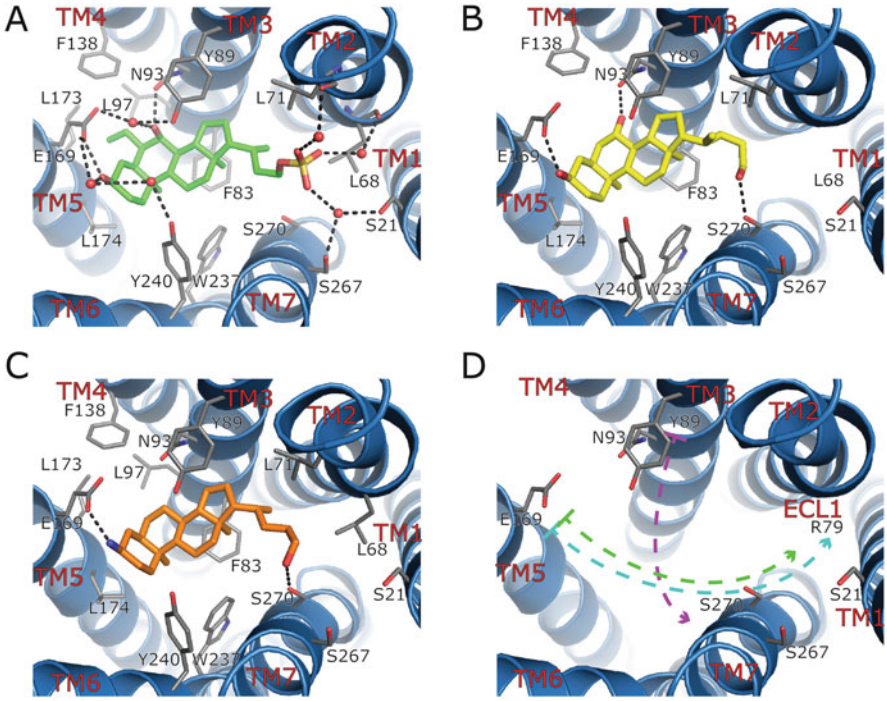

Fig. 6 (**a**, **b** and **c**) The predicted binding modes of three different BA derivatives to the GPBAR1 binding site: (**a**) INT-767 (green sticks) (D'Amore et al. 2014), (**b**) an alcohol analogue of UDCA (Sepe et al. 2014), (**c**) a LCA derivative having an amine group at the 3α-position (Di Leva et al. 2015). The GPBAR1 receptor is shown as light blue cartoon, while the ligand interacting residues are represented as grey sticks. The bridging water molecules are reported as red spheres. The GPBAR1 extracellular loops and nonpolar hydrogens are not displayed for clarity. (**d**) Schematic representation of the different orientations of GPBAR1 agonists as predicted by different research groups. The arrows represent the orientation of the molecules into the binding pocket, in which the tail and the head of the arrow represent the A ring and the side chain, respectively. The green arrow depicts the binding mode proposed by D'Amore et al. (2014), the magenta arrow that of Macchiarulo et al. (2013) and the cyan arrow that of Gertzen et al. (2015)

The first study that merits to be mentioned is that of D'Amore et al. (2014). Here, the authors built the 3D model of GPBAR1 using as template the adenosine A_{2A} GPCR, which was employed to elucidate the binding mode of the potent bile acid derivative INT-767 (Rizzo et al. 2010) through docking and MD calculations (Fig. 6a). In the proposed binding mode, the ligand is placed parallel to the phospholipids bilayer, with the A ring of the steroidal scaffolds contacting TM5 and the side chain interacting with TM1 and TM2 (D'Amore et al. 2014). The binding pose presents the structural features known to be essential for the activation of GPBAR1. In detail, these are (1) a H-bond with Glu169 and a water-mediated interaction with Tyr240 through the BA's 3α-hydroxyl group; (2) a H-bond with Asn93 and water-mediated interactions with Tyr89 and Glu169 by the BA's

7α-hydroxyl group; (3) the hydrophobic interaction of the BA's steroidal scaffold with residues such as Leu71, Phe83, Leu174 and Trp237; and (4) the H-bond interactions, direct or mediated by the water molecules present in the binding cavity, between the sulphate group in the BA's side chain and Ser21 on TM1 and Ser267 and Ser270 on TM7 (D'Amore et al. 2014). The proposed binding mode is in good agreement with previous mutagenesis data reporting a decrease of the binding affinity for BA ligands in the Asn93Ala, Glu169Ala and Tyr240Ala mutant forms of GPBAR1 (Macchiarulo et al. 2013). As proof of concept, D'Amore et al. designed new derivatives based on the proposed binding mode in which the ligand's side chain was prolonged to establish direct and stronger interactions through the sulphate group with the aforementioned serine residues. The resulting compounds indeed showed an increased affinity towards GPBAR1 if compared to INT-767 (D'Amore et al. 2014).

A further validation of the GPBAR1 3D model of D'Amore et al. is provided by the same authors in a following work in which they demonstrated that even ursodeoxycholic (UDCA) derivatives, presenting a 7β-hydroxyl group instead of the 7α-OH of INT-767, are able to bind the receptor's binding cavity establishing through the 3α- and 7β-hydroxyl groups H-bond interactions with Glu169 and Asn93, respectively. In this case, the ligand's flexible side chain points towards TM1, TM2 and TM7 where the carboxylate group interacts with Ser21 and Ser270 (Sepe et al. 2014) (Fig. 6b).

The relevance of having a reliable structure of the receptor to guide drug design is further demonstrated by the same group of authors in the work of Di Leva et al. in which they exploited the 3α-position on the steroidal scaffold to achieve GPBAR1 selective ligands over FXR (Di Leva et al. 2015). Specifically, they designed derivatives with an amine group instead of the hydroxyl at the 3α-position; the resulting compounds can thus form a strong salt bridge interaction with Glu169 in GPBAR1, while the same group, facing the positively charged His447, leads to poor affinity towards FXR (Di Leva et al. 2015) (Fig. 6c).

Using a different 3D model of GPBAR1 obtained through homology modelling and the use of rhodopsin in its inactive state as template (Macchiarulo et al. 2013), Macchiarulo et al. proposed the binding mode of the dual FXR/GPBAR1 agonist OCA and the semi-synthetic BA INT-777 (Pellicciari et al. 2009), a selective GPBAR1 ligand. Here, the ligand is placed in an orientation perpendicular to the lipid bilayer in which the compound is able to interact with TM3 (Fig. 6d) (Macchiarulo et al. 2013). In particular, the 3-hydroxyl group forms H-bonds with Asn93 and Tyr89, which the same authors identified as important for the binding of BAs to GPBAR1 through mutagenesis experiments. It is worth noting that in the binding model proposed by Macchiarulo et al., the ligand does not interact with Glu169, which is however confirmed by the same authors to be involved in the activation of the receptor according to the mutagenesis data (Macchiarulo et al. 2013).

Finally, a third 3D model of GPBAR1 was built by Gertzen et al. using the homology modelling approach based on multiple templates (Gertzen et al. 2015). Using such model, the authors elucidated the binding mode of the GPBAR1 agonist lithocholic acid taurine conjugate (TLC) and other 68 derivatives, which were validated

by mutagenesis experiments. In the predicted binding mode, the ligand is placed in the GPBAR1 binding site parallel to the membrane bilayer with its cholane scaffold close to TM5 and TM6 (Fig. 6d) (Gertzen et al. 2015). It is interesting to note that this binding mode is similar to that predicted by D'Amore et al. (2014) (Fig. 6d), although the two models were obtained using different templates. On the contrary, the ligand position in the receptor's binding site is rotated by about 180° if compared with that proposed by Macchiarulo et al. (Macchiarulo et al. 2013) (Fig. 6d). In more detail, in the binding mode proposed by Gertzen et al., the ligand interacts with Glu169, as also found by D'Amore et al., while it does not interact with Asn93, as in the case of the model of D'Amore et al. and by Macchiarulo et al., since in Gertzen's model, this residue points far from the binding site. Finally, in the binding mode of Gertzen et al., the long taurine-conjugated side chain of the ligand is placed in the receptor's pocket shaped by Ser21 on TM1 and Ser270 on TM7, in agreement with what is proposed by D'Amore et al., however, reaching a more external site where it forms H-bond interaction with Arg79 on the ECL1 (Fig. 6d) (Macchiarulo et al. 2013; D'Amore et al. 2014; Sepe et al. 2014; Di Leva et al. 2015; Gertzen et al. 2015).

In the recent years, the BAs scaffold has been largely investigated to achieve steroidal derivatives with different pharmacological profiles, from selective GPBAR1 agonists to dual agonists/antagonists of GPBAR1 and FXR (D'Amore et al. 2014; Sepe et al. 2016b; Carino et al. 2017) (see Sect. 3.3), useful in the treatment of lipid and glucose-related disorders. Some FXR agonists entered in Phase II clinical trial showed itching as the most disturbing side effect. Since GPBAR1 is the physiological mediator of pruritus, the development of GPBAR1 antagonists has attracted the attention to circumvent this limitation. Only few examples of BA derivatives as GPBAR1 antagonists have been reported so far (Sepe et al. 2016b; Li et al. 2017). In particular, Sepe et al. generated a library of cholane derivatives starting from the scaffold of the lithocholic acid (LCA). In more detail, the authors modified (1) the functionalization of the steroidal scaffold; (2) the stereochemistry of the A/B ring junction; and (3) the functionalization and the length of the ligand's side chain (Sepe et al. 2016b).

3.3 FXR/GPBAR1 Dual Modulators

In the previous paragraphs, we have described the general features of BAs for interacting preferentially with either FXR or GPBAR1. The reported findings highlight that the discovery of selective ligands of these receptors represents a challenging task for medicinal chemists due to the similar structural requirements of ligands interacting with their orthosteric binding sites. On the other hand, this fact has paved the way for the development of a large array of BA derivatives as FXR/GPBAR1 dual agonists. The most representative examples are the formerly cited OCA (Pellicciari et al. 2002) and INT-767 (Rizzo et al. 2010), which indeed share analogous structural features such as (1) the C24-5β-cholanoic acid scaffold; (2) the 3α- and a 7α-hydroxyl groups; and (3) the 6α-ethyl substituent. The two ligands only differ in the type of the terminal polar group on the side chain, which are

a carboxylic acid in OCA and a sulphate moiety in INT-767. As previously reported, such features allow these compounds to contact the amino acids that are important for the activation of both FXR and GPBAR1. In the following discussion, we consider the binding mode of INT-767 to FXR and GPBAR1 reported by D'Amore et al. (2014) (Figs. 5b and 6a). In detail, the 3α-OH group interacts with His447 in FXR and Glu169 in GPBAR1, while the 7α-OH forms H-bonds with Ser332 and Tyr369 in FXR and Asn93 and Tyr89 in GPBAR1 (Mi et al. 2003; D'Amore et al. 2014). The important role played by these interactions is supported by the experimental findings that both the hydroxyl functions, with the same stereochemistry, are required to achieve dual FXR/GPBAR1 agonists (Sepe et al. 2014, 2016a). Concerning the 6α-ethyl moiety, it occupies in both receptors an ancillary hydrophobic pocket, where it can form a number of van der Waals contacts that contribute to the ligand binding affinity. Finally, the terminal polar group on the side chain forms H-bonds with Arg331 in FXR and with the serine residues of TM1 and TM7 in GPBAR1 (Mi et al. 2003; D'Amore et al. 2014). Computational and experimental data suggest that this interaction network is conserved when the carboxylic/sulphate moiety of OCA/INT-767 is replaced by neutral polar groups such as an alcoholic function, also tolerating small changes in the side chain length (i.e. from C24 to C26 in the BA structure) (D'Amore et al. 2014; Festa et al. 2014). In fact, compounds resulting from such modifications typically maintain the dual FXR/GPBAR1 agonist activity. On the other hand, as anticipated in the previous sections, the introduction of additional hydroxyl groups such as β-OH at carbon 11 (Pellicciari et al. 2016), or ramification in the ligand's side chain (Pellicciari et al. 2009), can lead to ligands with selective activity profiles towards either FXR or GPBAR1.

4 Non-Bile Acid FXR and GPBAR1 Modulators

With the aim at achieving selectivity over the other bile acid receptors and avoiding pharmacokinetic issues related to the use of BA derivatives, medicinal chemists put efforts to develop non-BA compounds as FXR and GPBAR1 modulators. Among these are both natural and non-natural compounds that are endowed with pharmacological profiles varying from full and partial agonism to full antagonism. In the following paragraphs, we describe the binding mechanism of the non-BA ligands that have been disclosed by crystallographic and computational studies, providing valuable insight into the molecular basis of FXR and GPBAR1 regulation by non-BA molecules.

4.1 Non-Bile Acid FXR Agonists

The first X-ray structure of FXR with a non-BA compound was released in 2003 when the hFXR-LBD was solved in complex with fexaramine, a potent synthetic agonist of this receptor (Downes et al. 2003). The FXR/fexaramine complex revealed that this ligand binds to the orthosteric site of the receptor in a mode

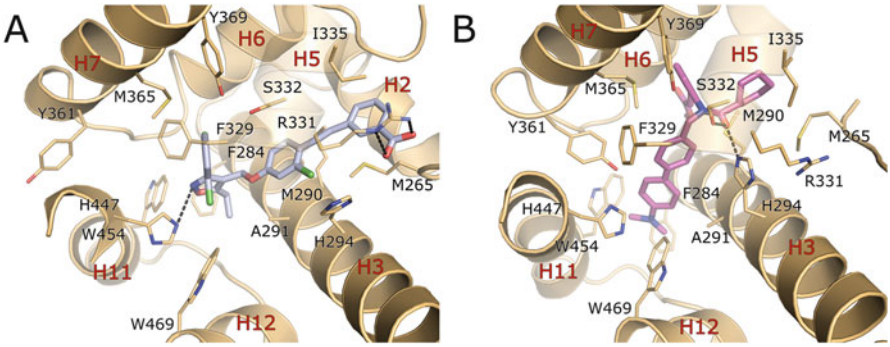

Fig. 7 The binding mode of (**a**) GW4064 (light blue sticks) (Akwabi-Ameyaw et al. 2008) and (**b**) fexaramine (violet sticks) (Downes et al. 2003) to the FXR-LDB. The receptor is shown as orange cartoon, with the ligand interacting amino acids represented as orange sticks. H8 and H10 and nonpolar hydrogens are not displayed for clarity. Hydrogen bonds are shown as dashed black lines

different from that of BA derivatives like OCA. In fact, at variance with this compound, fexaramine binds to FXR mainly through hydrophobic interactions, which altogether stabilize the receptor in its agonist conformation. The ligand can however form two H-bonds with the side chains of His294 and Ser332 through its amide group, which further contribute to the overall binding affinity (Fig. 7a). On the other hand, fexaramine is not able to contact Arg331 and His447 that instead play a major role in the binding of BAs to FXR, indicating that the activation state of this receptor can be modulated through different mechanisms. It is interesting to remark that, if compared to OCA, fexaramine induces some changes in the tertiary structure of the protein. In fact, in the FXR/fexaramine complex, H3 and H6 are notably shifted with respect to the FXR/OCA X-ray structure, whereas H2 is not solved. These evidences suggest that the receptor can undergo large fluctuations and the plasticity of its binding site, similar to other NRs, allows hosting ligands of different size and nature. Among these is GW4064, a potent and selective synthetic FXR agonist developed by GlaxoSmithKline (Maloney et al. 2000), which was co-crystallized in complex with FXR in 2008 (Akwabi-Ameyaw et al. 2008). The X-ray complex revealed that, at variance with fexaramine, the binding mode of GW4064 is superimposable with that of BA derivatives (Fig. 7b). In fact, similar to OCA, the carboxylic acid of GW4064 forms a salt bridge interaction with the guanidinium group of Arg331 in H5. Furthermore, the ligand isoxazole ring can interact with the protonated side chain of His447, stabilizing the cation-π interaction formed by this residue with Trp469 that is known to play a key role in the activation of the receptor. Differently from OCA, GW4064 cannot form H-bonds with Ser332 and Tyr369, which are involved in the FXR activation operated by BA derivatives. This observation indicates that the presence of the latter interactions is not necessary for the interaction of non-BA agonists with FXR. As observed for BAs and fexaramine, the binding mode of GW4064 is further stabilized by several lipophilic contacts. In particular, the isopropyl-isoxazole moiety occupies the aromatic cage

defined by H3, H11 and H12 where it forms π-stacking and van der Waals interactions with the side chains of residues such as Phe284, His447 and Trp454. Additional hydrophobic contacts are formed by the ligand's 2,6-dichloro phenyl ring with the side chains of Phe329, Met365 and Tyr369 and finally by the stilbene scaffold with residues such as Met265, Met290 and Phe336. The information gathered from the FXR/GW4064 X-ray complex has been extensively exploited to develop a set of derivatives with improved pharmacological properties. Crystallographic and computational investigations have demonstrated that these ligands are able to recapitulate the interaction pattern of the parent compound GW4064 (Akwabi-Ameyaw et al. 2008, 2009, 2011; Bass et al. 2009, 2011; Sepe et al. 2018), representing a successful case of structure-based rational lead optimization.

In addition to fexaramine and GW4064, other synthetic and semi-synthetic agonists with different scaffolds have been crystallized in complex with FXR, which include the tetrahydroazepinoindole compound FXR-450 (Flatt et al. 2009). Similar to fexaramine, FXR-450 binds to the receptor LBD mainly through hydrophobic contacts, although it can form H-bonds with Ser332 and Tyr369 through its ester and amide functions, respectively. As mentioned above, these interactions are not formed by GW4064; however they have been also reported for small-sized benzimidazolyl acetamides FXR agonists (Richter et al. 2011). This fact suggests that such contacts might be important for the FXR activation operated by low molecular weight non-BA ligands, assisting the ligand/receptor lipophilic interactions in the stabilization of the FXR agonist conformation. It is also interesting to note that some FXR-450 analogues, endowed with elongated branches, extend towards a solvent-exposed region defined by H2 and H6 (Lundquist et al. 2010), which approximately corresponds to the S2 site described by Gioiello et al. (Gioiello et al. 2011) and cited in Sect. 3.1. In particular, through a ligand-induced fit mechanism, the H2 undergoes a conformational rearrangement allowing the ligand to accommodate properly and activate the receptor (Lundquist et al. 2010). Finally, a steroidal glucocorticoid compound, mometasone furoate, has been recently proposed as FXR agonist; however a unique binding mode of this ligand in the receptor binding site has not been disclosed so far (Bijsmans et al. 2015).

4.2 Non-Bile Acid FXR Antagonists

At variance with the non-BA FXR agonists, few experimental and theoretical data have been reported on the binding mechanism of antagonist molecules to the FXR-LBD. Nonetheless, using the available information is possible to delineate the general features of FXR antagonism. Similar to other NRs such as the glucocorticoid (Schoch et al. 2010) and the oestrogen receptors (Brzozowski et al. 1997), FXR can be inactivated either by the so-called passive antagonists, which destabilize the receptor-coactivator complex, or through the "active" antagonists that favour the binding of corepressor peptides to the receptor. As final result, both lead to the decrease of the expression of the genes regulated by FXR (Shiau et al. 2002).

The first compound reported as FXR active antagonist was the promiscuous NRs ligand guggulsterone (GS) (Urizar et al. 2002; Burris 2004), which has been better characterized later on as a gene-specific modulator of FXR. In particular, this compound acts as FXR agonist or antagonist in dependence of the gene whose expression is regulated by FXR (Cui et al. 2003). These data suggest that a complex molecular recognition process might occur between GS and FXR. Computational studies hypothesized that GS can occupy the S2 site of the FXR-LBD, modulating the receptor conformational changes and in turn its affinity for coactivator and corepressor peptides (Meyer et al. 2005).

An example of FXR passive antagonism is represented by the marine sponge sesterterpene suvanine, whose binding mechanism to FXR was investigated by Di Leva et al. combining docking and MD simulations. These calculations indicated that suvanine binds to the orthosteric binding site of the FXR-LBD disrupting the crucial cation-π interaction between His447 and Trp469. This event induces H12 to assume a conformation unsuitable for the binding of the coactivator peptide (Fig. 8a) (Di Leva et al. 2013). Further insight into the mechanism of action of FXR passive antagonists has been provided by the crystal structure of the FXR-LBD in complex with the selective antagonist *N*-benzyl-*N*-(3-(tert-butyl)-4-hydroxyphenyl)-2,6-dichloro-4-(dimethylamino) benzamide (NDB), which is able to induce the homodimerization of the receptor. The NDB/FXR interaction occurs through a number of hydrophobic contacts, although the ligand can also form a H-bond with the side chain of His294 of H3 and water-mediated interactions with Tyr361 of H7 and His447 of H11 (Fig. 8b). It is important to remark that in the NDB-bound conformation, the structure of the FXR-LBD is largely different from that of the agonist form. In fact, in the antagonistic state, the hydrophobic C-terminal part of H11 is bent filling part of the ligand-binding pocket and moving H12 to occupy the binding site of coactivator and corepressor peptides in the other FXR monomer

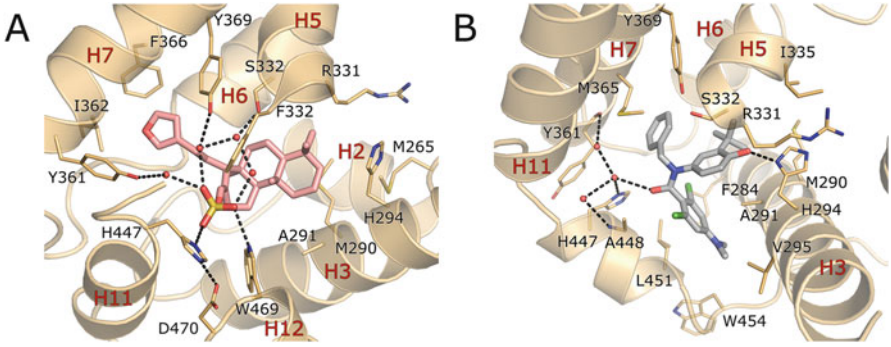

Fig. 8 The binding mode of (**a**) suvanine (pink sticks) (Di Leva et al. 2013) and (**b**) NDB (grey sticks) (Xu et al. 2015) to the FXR-LDB. The receptor is shown as orange cartoon, with the ligand interacting amino acids represented as orange sticks. H8 and 10 and nonpolar hydrogens are not displayed for clarity. Water molecules are depicted as red spheres. Hydrogen bonds are shown as dashed black lines

(Xu et al. 2015). A similar binding mechanism has been recently proposed for nonacidic oxadiazole and benzimidazole derivatives exhibiting promising FXR antagonist profiles (Teno et al. 2018; Festa et al. 2019).

4.3 Non-Bile Acid GPBAR1 Modulators

At variance with FXR, there exist only a couple of examples of non-BA GPBAR1 ligands. In particular, nomilin, a limonoid extracted from *Citrus* spp., is known to be effective in suppressing high-fat-diet-induced obesity and hyperglycaemia in mice by targeting the murine isoform of GPBAR1 (Sasaki et al. 2017). Prompted by these data, Sasaki et al. investigated its activity and binding mode in the receptor human isoform. Mutagenesis data identified Gln77, Arg80 and Tyr89 as crucial for the GPBAR1-nomilin interaction. In particular, the ligand lays in the receptor-binding pocket parallel to the lipid bilayer and is stabilized by H-bond interactions with these three residues, thus revealing a different binding mode with respect to those of the BA derivatives (Sasaki et al. 2017).

A second example of non-BA GPBAR1 agonists is provided in a very recent work of Di Leva et al. who performed a structure-based drug design study that led to the identification of a new series of non-steroidal selective GPBAR1 agonists endowed with a ((1, 2, 4-oxadiazol-5-yl)pyrrolidin-3-yl)ureidyl scaffold (Di Leva et al. 2019). Combining docking and MD simulations, the authors revealed the binding mode of the most promising compound of the series (Fig. 9). Here, the ligand pyrrolidinyl ring engages a salt bridge with the side chain of Glu169 and a H-bond with Asn93, while the phenyl-oxadiazole moiety deepens in the lipophilic pocket formed by the side chains of residues such as Leu85, Phe96, Leu100, Leu173, Leu174 and Trp237 and Tyr240. In addition, the cyclohexyl ring occupies the small hydrophobic cleft defined by Leu68 and Leu71 and Phe96, already found

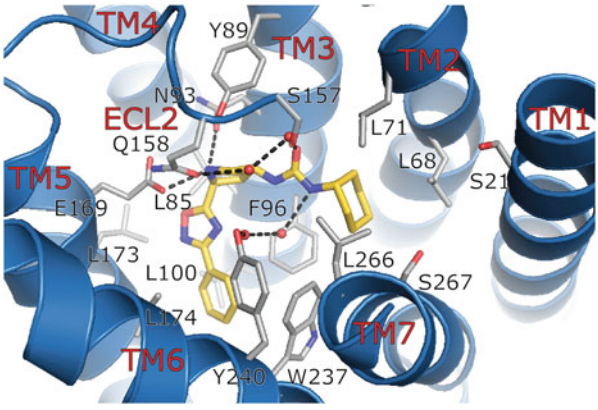

Fig. 9 The predicted binding mode of the non-BA GPBAR1 agonist discovered by Di Leva et al. (2019). The ligand and interacting residues are represented as golden and grey stick, respectively, while the GPBAR1 receptor is shown as light blue cartoon. The bridging water molecules are depicted as red spheres. The GPBAR1 extracellular loops and nonpolar hydrogens are not displayed for clarity

to interact with some BA derivatives (D'Amore et al. 2014; Sepe et al. 2014; Di Leva et al. 2015), while the ureidic group forms water-mediated contacts with Ser157, Gln158 and Tyr240.

The first example of non-BA GPBAR1 antagonist has been instead reported by Li et al. who characterized both in vitro and in vivo the antagonist activity of triamterene towards GPBAR1 (Li et al. 2017). Unfortunately, no structural data on the binding mode of both BA and non-BA antagonists to GPBAR1 are available so far, thus hampering rational drug design studies. In this context, the group of Prof. Limongelli is modelling using state-of-the-art MD techniques the GPBAR1 structure in its inactive conformation (i.e. antagonist bound) starting from its active structure (i.e. agonist bound). The availability of the GPBAR1 structure in its inactive state will be instrumental to guide the drug design of antagonist compounds with tailored activity towards the receptor as performed in the last decade in the case of the GPBAR1 agonists.

5 Conclusions and Perspectives

In this chapter, we have provided a detailed description on the structure of the BA receptors FXR and GPBAR1 and their interaction with both BA and non-BA modulators. We have shown that in the case of FXR, many crystallographic data are available, which reveal the ligands' chemical features required to bind and activate/inactivate the receptor. Experimental findings on FXR have been, however, integrated by extensive computational studies that have allowed the researchers to extend the structure-activity relationships studies for the known ligands and to define with more accuracy the molecular mechanisms underlying the modulation of this nuclear receptor. At variance with FXR, no experimental structure of GPBAR1 is available so far. However, using bioinformatics and computational methodologies, also with the aid of mutagenesis data, three tridimensional models of this receptor have been reported. These structures have been validated in a number of studies and exploited to define the molecular basis of the GPBAR1/ligand interaction. The findings gathered from both theoretical and experimental studies on FXR and GPBAR1 have been successfully used to afford new compounds endowed with different activity and selectivity profiles towards the two receptors. At this regard, it is important to underline that the development of selective FXR and GPBAR1 is challenging due to the similar structural features of the ligand binding site of the two receptors. This evidence demands the aid of computational methodologies such as homology modelling, molecular docking and MD in the rational drug design of novel compounds with tailored activity towards either receptor.

In spite of the large number of data available, some aspects of the regulation of FXR and GPBAR1 remain unclear. For instance, no structural information on the binding mode of antagonist ligands to GPBAR1 is available so far. Advance in this sense is awaited from ongoing theoretical studies that aim at defining the antagonist-bound structure of GPBAR1 and characterizing the transition of the receptor from its active to its inactive state. The tridimensional GPBAR1 structure in the inactive

conformation might indeed guide the drug design of brand new selective antagonists of this receptor, thus providing potential drug candidates for itching but also chemical tools for a deeper investigation of the biological role and function of this receptor. In the case of FXR, further insight into its activation mechanism is expected by analysing in more detail the receptor interaction with partial agonist ligands. In fact, at variance with full agonist and antagonist ligands, only few experimental and theoretical studies have been reported so far on the binding mode of partial agonists to FXR, and the effects of their binding on the different receptor conformational states remain poorly understood. In this context, the recent identification of the allosteric site S2 has provided valuable hints for the design of novel FXR modulators where we can foresee that the computational methodologies will play a central role.

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Chemistry and Pharmacology of GPBAR1 and FXR Selective Agonists, Dual Agonists, and Antagonists

Simona De Marino, Carmen Festa, Valentina Sepe, and Angela Zampella

Contents

Abstract

In the recent years, bile acid receptors FXR and GPBAR1 have attracted the interest of scientific community and companies, as they proved promising targets for the treatment of several diseases, ranging from liver cholestatic disorders to metabolic syndrome, inflammatory states, nonalcoholic steatohepatitis (NASH), and diabetes.

Consequently, the development of dual FXR/GPBAR1 agonists, as well as selective targeting of one of these receptors, is considered a hopeful possibility in the treatment of these disorders. Because endogenous bile acids and steroidal ligands, which cover the same chemical space of bile acids, often target both receptor families, speculation on nonsteroidal ligands represents a promising and innovative strategy to selectively target GPBAR1 or FXR.

S. De Marino · C. Festa · V. Sepe · A. Zampella (✉)
Department of Pharmacy, University of Naples "Federico II", Naples, Italy
e-mail: angela.zampella@unina.it

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In this review, we summarize the most recent acquisition on natural, semisynthetic, and synthetic steroidal and nonsteroidal ligands, able to interact with FXR and GPBAR1.

Keywords
Bile acid receptors · Farnesoid X receptor (FXR) · G-protein-coupled receptor (GPBAR1) · Natural ligands · Semisynthetic ligands · Synthetic ligands

Abbreviations

ANIT	Alpha-naphthylisothiocyanate
BSEP	Bile salt export pump
CDCA	Chenodeoxycholic acid
DIO	Diet-induced obesity
HFD	High-fat diet
NAFLD	Nonalcoholic fatty liver disease
NASH	Nonalcoholic steatohepatitis
PBC	Primary biliary cirrhosis
SHP	Small heterodimer partner
UDCA	Ursodeoxycholic acid

1 Introduction

Liver and metabolic disorders result from a cluster of diverse physiological, biochemical, clinical, and metabolic factors that greatly increase an individual's probability for developing NASH, atherosclerotic cardiovascular disease, type 2 diabetes, as well as other liver cholestatic conditions such as primary sclerosing cholangitis (PSC).

Because these disorders represent a growing global public health problem, and current therapies expose the patients to several side effects, there is increased interest in developing new pharmacological tools that could provide new opportunities in the treatment of complex metabolic disorders in which several target pathways are involved.

Among different targets, the bile acid receptors FXR and GPBAR1 represent promising pharmacological targets in the management of such diseases, with potential effectiveness of their small-molecule modulators for the treatment of metabolic and enterohepatic disorders.

The primary aim of this review is to provide the reader with an overview on natural, semisynthetic, and synthetic ligands belonging to steroidal and nonsteroidal chemical classes, able to interact with these two receptors. Since this topic has been recently treated in literature (Sepe et al. 2015a, 2018; Xu 2016; Carotti et al. 2014;

Goldstein and Levy 2018; Merk et al. 2012), we will describe exclusively the most recent acquisitions.

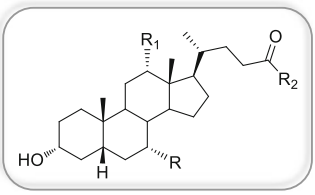
2 Natural FXR and GPBAR1 Modulators

2.1 Natural FXR Agonists (Fig. 2)

After FXR de-orphanization by bile acids (Makishima et al. 1999; Parks et al. 1999; Wang et al. 1999), research interests have been especially focused on steroids and terpenoids, hydrophobic molecules widely distributed within vegetal and marine realm, and sharing with bile acids several key structural features in their molecular architecture (Fig. 1 shows the family of mammalian bile acids).

Lu and co-workers reported hedragonic acid (24-nor-3-oxo-12-oleanen-28-oic acid), as the first natural oleanane-type triterpene selective FXR agonist with anti-inflammatory activity and liver protection (Lu et al. 2018). Hedragonic acid has been isolated from the stem and root of *Celastrus orbiculatus* Thunb. (COT), a woody vine of the Celastraceae family. It showed a potent effect in inducing the recruitment of the classic FXR coactivators (SRC-1, SRC-2, and SRC-3 (steroid receptor coactivator-1, steroid receptor coactivator-2, and steroid receptor coactivator-3)) in Alfa Screen assay and functional potency in cell-based transactivation assays. Moreover, in vivo experiments demonstrated that this compound exerts therapeutic effects on liver injury induced by acetaminophen overdose and in hepatic inflammation, suggesting that it might be a potential lead in liver diseases.

Other triterpenes able to activate FXR are alisol derivatives. These compounds were isolated from *Alisma orientalis*, a well-known traditional medicinal plant used



CDCA	R=OH	R_1=H	R_2=OH
CA	R=OH	R_1=OH	R_2=OH
DCA	R=H	R_1=OH	R_2=OH
LCA	R=H	R_1=H	R_2=OH
TLCA	R=H	R_1=H	R_2=$NHCH_2CH_2SO_3Na$

FXR agonism

GPBAR1 agonism

Fig. 1 Endogenous bile acids

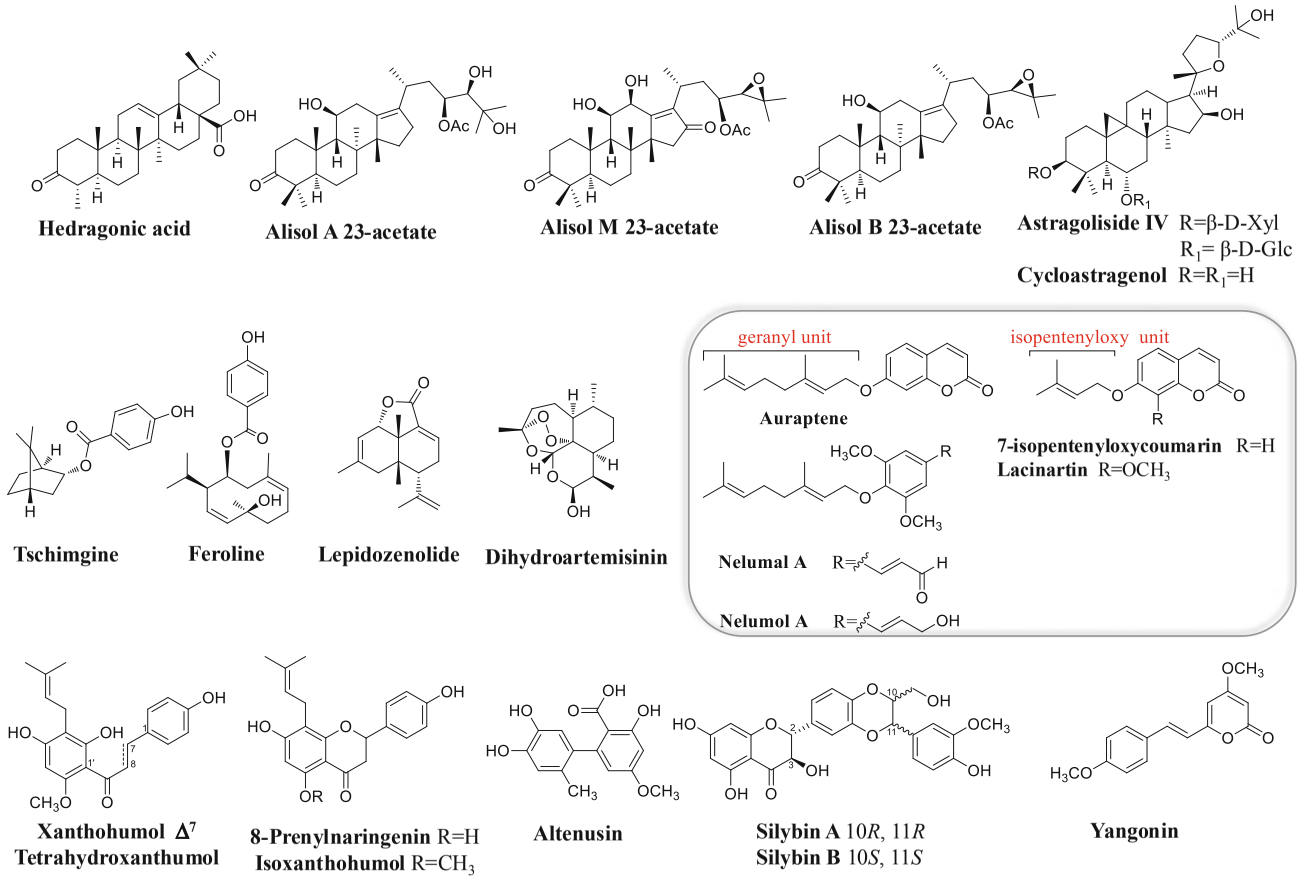


Fig. 2 Natural FXR agonists

for a broad range of pharmacological effects such as antidiabetic, antihepatitis, and antidiuretics (Peng et al. 2003; Hu et al. 2008). The chemical composition of this medicinal plant consists mainly in guaiane-type sesquiterpenes and protostane-type triterpenes such as alisol derivatives. Among them, alisol A 23-acetate and alisol M 23-acetate feature a steroidal scaffold with a keto group at C-3 mimicking the 24-carboxylic group of CDCA (chenodeoxycholic acid), the endogenous FXR agonist. Due to this structural similarity, alisol A 23-acetate and alisol M 23-acetate were evaluated for their ability to transactivate FXR (Lin 2012), and the results showed that both these two triterpenes exhibit an agonistic activity equipotent to CDCA.

From *Rhizoma alismatis*, a medicinal plant widely used as a traditional Chinese medicine for a long time (Wang et al. 2004), alisol B 23-acetate was isolated. This compound promotes liver regeneration via FXR activation on a mice model of partial hepatectomy (Meng et al. 2014), ameliorating hepatic triglyceride accumulation in animal model of NASH as well as liver injury in ANIT (alpha-naphthylisothiocyanate)-induced intrahepatic cholestasis (Meng et al. 2015, 2017).

Astragalus membranaceus Bunge is the botanical sources of Astragali Radix, an herbal medicine used in China for the treatment of several diseases such as diabetes, hyperlipidemia, atherosclerosis, and cancer (Li et al. 2017). Among its constituents the saponin astragaloside IV (3-*O*-β-D-xylopyranosyl-6-*O*-β-D-glucopyranosyl-cycloastragenol), which, in vivo, is rapidly metabolized to the corresponding aglycone cycloastragenol, has recently been proved as FXR agonist. Consistent with these results, cycloastragenol treatment in diet-induced obesity (DIO) mice considerably reduced fat accumulation in liver, lowered blood glucose and serum triglyceride levels, and reconstituted the liver bile acid composition. In addition, cycloastragenol improved hepatic steatosis, resulting to be potentially effective against NAFLD (nonalcoholic fatty liver disease) and NASH (Gu et al. 2017).

In 2017, Zheng et al. identified tschimgine and feroline, as a novel class of FXR modulators (Zheng et al. 2017b). These two natural terpenoids, featuring a molecular scaffold different from CDCA, potently activated FXR in a concentration-dependent manner ($EC_{50} \approx 0.56$ μM). Crystal structure of FXR complexed with tschimgine or feroline revealed that the above compounds bind FXR in a different, unexpected, previously unreported hydrophobic pocket. Moreover, these compounds induced conformational changes in the activation function 2 (AF-2) surface, suggesting a partial agonism of FXR.

In the mammalian one-hybrid and transient transfection reporter assays, lepidozenolide, a sesquiterpene isolated from *Lepidozia fauriana*, has been recently identified as FXR agonist. Docking studies on lepidozenolide in FXR-LBD revealed that the carbonyl group H-bonds with His 447 of helix 11, stabilizing FXR in the active conformation. However, this compound made less hydrophobic and hydrophilic interactions than CDCA in FXR-LBD, explaining its slightly weaker agonism compared to the endogenous activator (Lin 2015).

Artemisinin, a δ-sesquiterpene lactone endoperoxide isolated from *Artemisia annua* L., is largely used for the treatment of malaria (Guo 2016). Recently, dihydroartemisinin, the semisynthetic derivative of artemisinin, was demonstrated

as FXR ligand (Xu et al. 2017). Dihydroartemisinin attenuates hyperlipidemia and reduces hepatic steatosis through the regulation of lipogenesis and lipolysis genes, thus representing a promising candidate for liver disorders.

In 2012, Epifano et al. reported the pharmacological evaluation of a series of natural and semisynthetic oxyprenylated and azoprenylated phenylpropanoids (Epifano et al. 2012). Three compounds, nelumol A, nelumal A, and auraptene, were demonstrated FXR agonists with a potency comparable to CDCA. Having synthetized a library of derivatives, the authors also demonstrated that the geranyl-substituted derivatives were more active than those featuring an isopentenyloxy side chain, such as 7-isopenthenyloxycoumarin and lacinartin. Nelumal A and nelumol A differ each other for the presence, in nelumal A, of an α,β-unsaturated conjugated aldehyde on the phenylpropanoid core. Probably, the presence of this structural feature is responsible for the best FXR agonistic activity of nelumal A. The three compounds were also found to have a large spectrum of pharmacological effects including, for auraptene, anti-inflammatory, anticancer, antifungal, antiprotozoal, and antioxidant activities (Genovese and Epifano 2011). In addition, auraptene was found also to act as a dual activator of PPARα and PPARγ (peroxisome proliferator-activated receptor alpha and gamma) (Takahashi et al. 2008; Kuroyanagi et al. 2008).

In 2005, Nozawa reported that xanthohumol, a prenylflavonoid isolated from beer hops *Humulus lupulus* L., ameliorated hyperlipidemia disorders, and this effect was correlated bona fide to its agonistic activity on FXR (Nozawa 2005). In order to gain insight into the molecular mechanisms of FXR modulation by prenylflavonoids, Yang et al., using a combination of HDX-MS (hydrogen deuterium exchange mass spectrometry), fluorescence titration, and molecular docking studies, have tested four prenylflavonoids (xanthohumol, isoxanthohumol, 8-prenylnaringenin, and a semisynthetic derivative tetrahydroxanthohumol) describing the conformational changes of FXR-LBD induced by these molecules (Yang et al. 2016).

Altenusin, isolated from the culture broths of *Penicillium sp.* (Nakanishi et al. 1995), represents a new chemotype of nonsteroidal FXR agonist. Evaluation on luciferase reported assay showed that this compound has a potency and efficacy similar to those of CDCA. In addition, when incubated in hepatocytes isolated from WT (wild type) mice, altenusin induced the expression of SHP (small heterodimer partner), BSEP (bile salt export pump), and SP-B1 genes and suppressed the expression of CYP7A4, a gene negatively regulated by FXR. Further in vivo experiments disclosed that administration of altenusin to HFD (high-fat diet)-induced obese mice produced metabolic benefits, such as significant reductions in body weight and fat mass, alleviation of dyslipidemia and insulin resistance, and reversal of hepatic steatosis. These findings underline altenusin as a potential lead for the treatment of metabolic disorders (Zheng et al. 2017c).

Silymarin, the herbal extract obtained from the fruits and seeds of *Silybum marianum*, has been used for thousands of years as a remedy for liver and biliary tract diseases. The major bioactive constituent has been recently identified in the flavonolignan silybin, isolated in two diastereomeric forms, silybins A and B, which

differ in the configuration at C-10 and C-11 on the 1,4-benzodioxane ring (Bijak 2017). Both silymarin and silybins are able to bind and activate FXR and to inhibit NF-kB signal pathway. Moreover, this herbal extract reshapes the composition of liver BAs pool and attenuates hyperlipidemia and insulin resistance in HF diet-fed C57BL/6 mice (Abenavoli et al. 2010). In addition, administration to DIO mice resulted in several benefits in insulin resistance, hyperlipidemia, and inflammation progress, reaffirming the usefulness of this extract for the treatment of liver diseases, such as hepatitis and cirrhosis (Gu et al. 2016).

Very recently, yangonin, a kavalactone isolated from a perennial tropical shrub, was identified through a virtual screening campaign. As FXR agonist, yangonin regulates bile acid homeostasis increasing their efflux from the liver as well as decreasing their biosynthesis. As consequence, the above compound promotes liver regeneration and attenuates hepatotoxicity induced by ANIT in animal model (Gao et al. 2018).

2.2 Natural FXR Antagonists

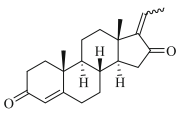
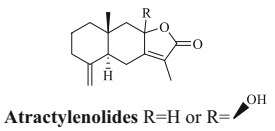
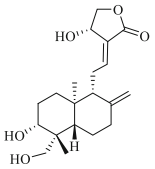
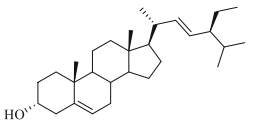
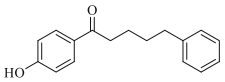
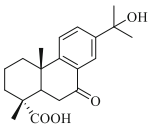
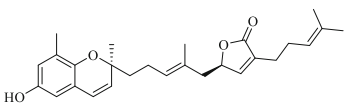
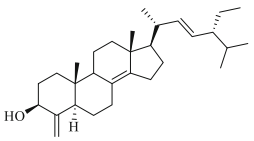
Starting from the identification of guggulsterone, a component of the resin extract of the tree *Commiphora mukul*, as the first example of nonselective natural FXR antagonist (Wu et al. 2002; Cui et al. 2003; Burris et al. 2005), several sterols with promising pharmacological behavior have been identified from plants and marine organisms. Chemistry and pharmacology of these ligands have been extensively discussed in several recent contributes (D'Auria et al. 2012; Sepe et al. 2015a, b; Huang et al. 2014; Fiorucci et al. 2012), and therefore the above topic will be not covered in this article. Table 1 summarizes the chemical structures and the natural sources of the most promising antagonists isolated so far from nature. As illustrated, with few exceptions, a large number of compounds cover the wide chemical space of steroidal scaffolds.

2.3 Natural GPBAR1 Agonists (Fig. 3)

While several FXR modulators have been identified from natural sources, only few examples of natural GPBAR1 ligands have been reported so far with the main contribution in the discovery of small-molecule modulators resulting from medicinal chemistry.

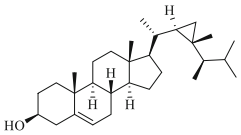
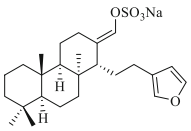
Among the first examples of natural GPBAR1 ligand, oleanolic acid, an active triterpene from *Olea europaea*, resulted able to activate the receptor with an EC_{50} comparable with that of LCA (lithocholic acid, the endogenous ligand of GPBAR1) (Sato et al. 2007). Further pharmacological studies demonstrated that oleanolic acid exerts antidiabetic effect in mice fed a high-fat diet improving glucose tolerance and increasing insulin release by pancreatic β-cell (Kumar et al. 2012). Other pentacyclic triterpene carboxylic acids, such as ursolic, quinovic, corosolic, betulinic, and maslinic acids, widely distributed in plant species, were recently demonstrated

Table 1 FXR antagonists isolated from natural sources

Compounds	Natural sources	References
 **E, Z Guggulsterone**	Tree *Commiphora mukul*	Wu et al. (2002)
 **Atractylenolides** R=H or R=	Medicinal plant *Atractylodes macrocephala*	Tsai et al. (2012)
 **Andrographolide**	Medicinal plant *Andrographis panniculata* (Burm.f.) *Nees*	Liu et al. (2014a)
 **Stigmasterol**	Various medicinal plants	Carter et al. (2007)
 **Daphneone**	Flowering plant *Daphne odora* Thunb	Diao et al. (2018)
 **Compound 37**	Plant *Abies georgei*	Diao et al. (2018)
 **Tuberatolide B**	Marine tunicate *Botryllus tuberatus*	Choi et al. (2011)
 **Theonellasterol**	Marine sponge *Theonella swinhoei*	Sepe et al. (2012) Renga et al. (2012)

(continued)

Table 1 (continued)

Compounds	Natural sources	References
 **Gorgosterol**	Soft coral *Sinularia* sp.	Putra et al. (2012)
 **Suvanine**	Marine sponge *Coscinoderma mathewsi*	Di Leva et al. (2013)

selective GPBAR1 ligands (Lo et al. 2016, 2017; Ladurner et al. 2017), with a beneficial GPBAR1 mediate effect in inducing GLP-1 secretion from L-cells.

Similarly, the triterpenoid saponin glycyrrhizic acid, extracted from the roots of the genus *Glycyrrhiza* commonly known as licorice, has been recently demonstrated a GPBAR1 agonist, also increasing plasma GLP-1 secretion in type 1-like diabetic rats (Wang et al. 2017a).

Belonging to limonoid triterpene subfamily, obacunone and nomilin showed beneficial effects in suppressing obesity and hyperglycinemia in mice due to their agonistic activity on GPBAR1 (Horiba et al. 2015; Ono et al. 2011).

Finally, Kirchweger and co-workers disclosed farnesiferol B and microlobidene, two sesquiterpene coumarins isolated from the gum resin of *Ferula assa-foetida* L., as potent GPBAR1 agonists (Kirchweger et al. 2018).

3 Synthetic Ligands

In the wide context of therapeutically application of FXR or GPBAR1 modulators, several ligands have been prepared and tested for affinity, efficacy, and selectivity with the identification of promising hit compounds with pharmacological profiles ranging from selective to dual modulation.

Two different approaches have been followed: (a) huge medicinal chemistry modification of bile acid scaffolds and in parallel and (b) identification of nonsteroidal chemotypes, which have also encountered high success from a therapeutic point of view.

3.1 Semisynthetic Bile Acid Derivatives (Fig. 4)

In recent years, the chemical manipulation on bile acid (BA) scaffold, with the aim to improve potency, efficacy, and metabolic stability, afforded many hit compounds

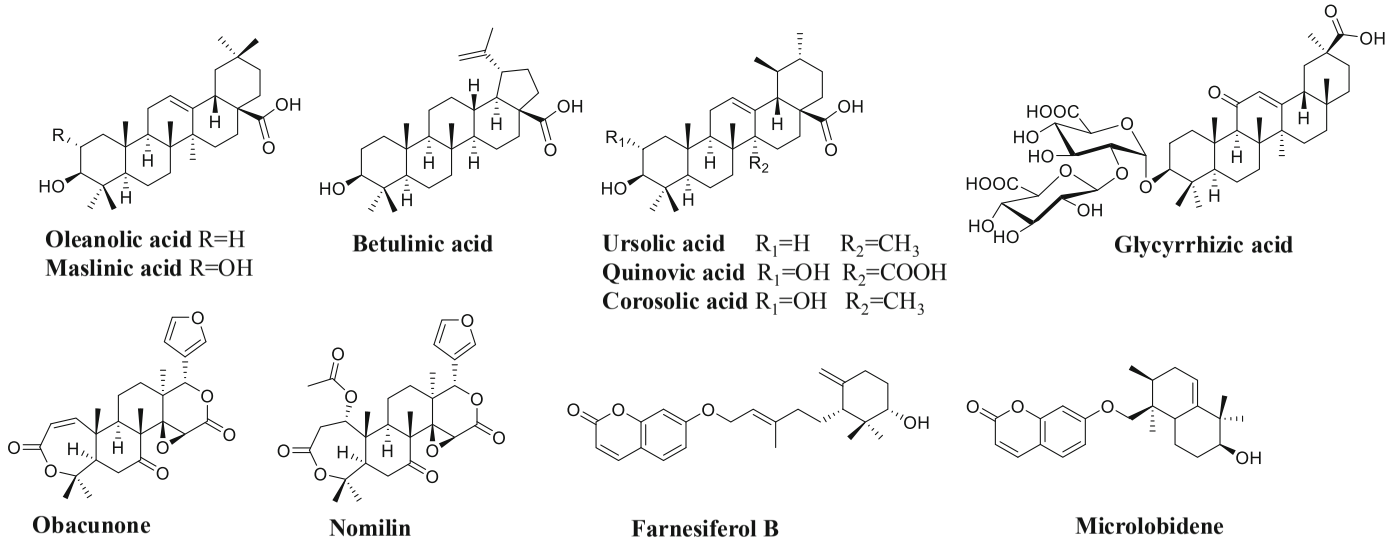
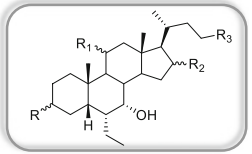
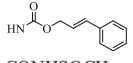
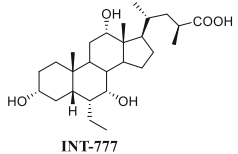


Fig. 3 Natural GPBAR1 agonists

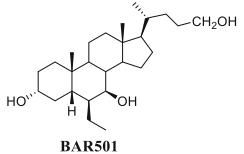


Compound	R	R_1	R_2	R_3	FXR In vitro assay[a]	FXR Transactivation assay[b]	GPBAR1
6-ECDCA/OCA/INT-747	,,,OH	H	H	COOH	0.099	0.5	0.9
TC-100	,,,OH	◢OH	H	COOH	0.14	nr[c]	>100
6α-ethyl-16-epi-avicholic acid	,,,OH	H	◢OH	COOH	11.5	nr[c]	0.65
BAR704	H	H	H	COOH	-	0.95	-
BAR710	◢OH	H	H	COOH	-	1.3	-
1	◢N_3	H	H	COOH	-	0.846	-
BAR502	,,,OH	H	H	OH	-	2	0.4
BAR504	,,,OH	H	H	CH_2OH	-	488.5 %[d]	103 %[d]
INT-767	,,,OH	H	H	OSO_3Na	0.03	0.01	0.47
2	,,,OH	H	H	CH_2OSO_3Na	-	1	0.2
3	,,,OH	H	H	NH_2	-	-	1.32
4	,,,OH	H	H		0.15	1	55
5	,,,OH	H	H	$CONHSOCH_3$	0.05	nr[c]	-

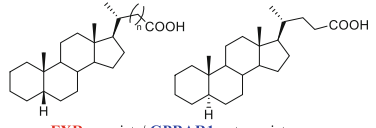
[a]EC_{50} values (μM) were calculated using an Alpha Screen assay; [b]Transactivation assays on HepG2 cells. EC_{50} values (μM) were calculated from at least three experiments; [c]nr=not reported; [d]BAR504 activity is measured as % of activation respect to CDCA set as 100%



INT-777
selective **GPBAR1** agonist
EC_{50} = 0.8 mM



BAR501
selective **GPBAR1** agonist
EC_{50} = 1.03 mM



FXR agonists/ **GPBAR1** antagonists
n = 0 **6**
n = 1 **7**
8

Fig. 4 Semisynthetic bile acid derivatives

with promising pharmacological profiles. Among bile acids, surely the privileged molecule has been recognized in CDCA, the most potent endogenous FXR activator and the results obtained in terms of SAR can be summarized as follows:

1. 6α-alkyl group is a key structural element in increasing FXR potency.
2. The hydroxyl group at C-3 has little influence on FXR agonistic activity.
3. The hydroxyl group at 7α-position is pharmacophoric and critical for the affinity to FXR.
4. The side chain tolerates significant structural variations in the length and in the nature of the end group.

Figure 4 summarizes the wide modification so far performed on CDCA and the pharmacological profiles in term of FXR/GPBAR1 activity.

In this frame, the semisynthetic 6α-ethylchenodeoxycholic acid (6-ECDCA/INT-747/obeticholic acid/OCA/OCAliva) has emerged as the most potent semisynthetic FXR agonist with an EC_{50} value of 0.099 μM in FRET (fluorescence resonance energy transfer) assay (Pellicciari et al. 2002). OCA has been approved for the treatment of UDCA (ursodeoxycholic acid)-resistant patients in PBC (primary biliary cirrhosis) (Mason et al. 2010) and represents the first FXR ligand to be progressed in Phase III clinical trials on NASH patients (Neuschwander-Tetri et al. 2015).

Indeed, severe and adverse drug effects emerged, such as pruritus (approximately 80% of treated patients), and increased low-density lipoprotein cholesterol levels that limited its market application. One of the molecular mechanisms underlying the pruritus observed with OCA administration could be imputable to OCA residual activity toward GPBAR1 (Maruyama et al. 2002; Pellicciari et al. 2016), recently demonstrated bona fide the physiological mediator of itching in mice (Alemi et al. 2013).

The introduction of a hydroxyl group on ring C, particularly at C-11β, produced a potent and selective FXR agonist, TC-100 endowed with improved physicochemical profile (Pellicciari et al. 2016).

Of interest, the introduction of the hydroxyl group on ring D increased GPBAR1 activity with 6α-ethyl-16-epi-avicholic acid a potent GPBAR1 agonist ($EC_{50} = 0.65 \pm 0.03$ μM, efficacy 120%) with a residual activity toward FXR (Pellicciari et al. 2012).

FXR selectivity was also targeted with removal, isomerization, or substitution at C3-hydroxyl group generating BAR704, BAR710, and compound **1**, potent FXR agonists in transactivation assays (Sepe et al. 2016a; Festa et al. 2017). Noteworthy, in contrast to OCA, which also transactivates GPBAR1 with an EC_{50} of 0.9 μM, BAR704 was proved to be a weak GPBAR1 antagonist with an IC_{50} of 9.5 μM. Administration to CCl_4 mice reduced the severity of liver damage and fibrosis with beneficial effects on liver microcirculation and fibrotic endothelial dysfunction by targeting TGFβ-SMAD3 pathway in hepatic stellate cells (HSCs). Moreover, in the same animal model, BAR704 shifts liver macrophage polarization from the pro-inflammatory (M1) phenotype to the anti-inflammatory M2 phenotype that is

characterized, among other markers, by increased expression of counter-regulatory genes such as IL-10 (Carino et al. 2018).

Modification at the side chain of 6-ECDCA produced several hit compounds with pharmacological activity ranging from selective modulation on FXR or GPBAR1 to dual modulation. Dual modulation is extremely useful in the treatment of diabetes and in the prevention of obesity's progression, NAFLD, and atherosclerosis.

Introduction of the hydroxyl or the sulfate group at C-24 or C-23 position on 6α-ethylcholane scaffold invariably produces dual FXR/GPBAR1 agonists such as BAR502 and BAR504 (Festa et al. 2014), and the corresponding sulfated derivatives, INT-767 (Rizzo et al. 2010) and compound **2** (D'Amore et al. 2014), with INT-767 and BAR502 the most advanced compounds in preclinical trials.

INT-767 has been profiled in different animal models, decreasing inflammation and fibrosis and improving metabolism (Moris et al. 2017; Rizzo et al. 2010; Roth et al. 2018; Jadhav et al. 2018). In two ethanol binge animal models, INT-767 was proved effective in reducing acute and chronic ethanol-induced steatosis and inflammation in mice reducing liver fatty acid synthase protein expression and modulating interleukin-1β mRNA expression (Iracheta-Vellve et al. 2018).

In rat model of NASH, INT-767 treatment significantly alleviates liver damage, restores the lipid and glucose metabolism, and attenuates insulin resistance and the pro-inflammatory response (Hu et al. 2018). Finally, in a rabbit model of HFD-induced metabolic syndrome, it reduces hypercholesterolemia, improves insulin resistance, and prompts preadipocyte differentiation toward a metabolically healthy phenotype ameliorating liver histological alterations (Hu et al. 2018; Comeglio et al. 2018).

BAR502, a recently discovered non-bile acid, steroidal, dual FXR/GPBAR1 ligand, represents a promising hit compound in the treatment of NASH. In a murine model of HFD, 9 weeks treatment with BAR502 promotes the browning of adipose tissue and the induction of cholesterol excretion from hepatocytes with a consequent amelioration of hepatic steatosis and reversion of steatohepatitis and fibrosis (Carino et al. 2017a).

Moreover, in two-mouse model of cholestasis, BAR502 modulates the liver expression of canonical FXR target genes including OSTα (organic solute transporter α), BSEP, SHP, and MDR1, increases survival, and attenuates serum alkaline phosphatase levels, without inducing pruritus (Cipriani et al. 2015).

Substitution of the carboxylic tail with amino (compound **3**) or carbamate groups led to the discovery of high efficacious and potent FXR agonists, such as 23-*N*-(carbocinnamyloxy)-3α,7α-dihydroxy-6α-ethyl-24-nor-5β-cholan-23-amine (**4**) (Gioiello et al. 2011). Therefore, amidation of the carboxylic group furnished compound **5** (Xiao et al. 2017) endowed with good pharmacokinetic properties and efficacy compared to 6-ECDCA.

On the other hand, 23(*S*)-methyl substitution on the side chain afforded INT-777, a potent and selective GPBAR1 agonist. INT-777-mediated GPBAR1 activation has been claimed as a promising approach to type 2 diabetes mellitus (Yu et al. 2015). Administration of INT-777 to db/db mice improves pancreatic β-cell proliferation, insulin synthesis and release, and glucose tolerance along with weight loss, and

stimulates GLP-1 synthesis from pancreatic α-cells under hyperglycemic conditions (Kumar et al. 2016). In addition, INT-777 stimulates GLP-1 release from enteroendocrine L-cells, increases energy expenditure, reduces hepatic steatosis, stimulates bile flow, gallbladder filling, and relaxation (Thomas et al. 2009), and inhibits macrophage inflammation and atherosclerosis (Pols et al. 2011). Very recently, INT-777 was also demonstrated as a useful approach in the treatment of lung diseases such as pulmonary arterial hypertension and idiopathic pulmonary fibrosis (Comeglio et al. 2017), with neuroprotective effects against LPS-induced cognitive impairment, neuroinflammation, apoptosis, synaptic dysfunction in mice, and against amyloid-beta-induced neurotoxicity (Wu et al. 2019; Wua et al. 2018).

Modification on the side chain in the length and in the nature of the end group and on the tetracyclic core of bile acid scaffold afforded BAR501 (Festa et al. 2014), the first example of C-6β-substituted UDCA derivative with potent and selective GPBAR1 activity.

In a rodent model of NASH, BAR501 reversed insulin resistance, ameliorates liver histology, and increases the weight of epWAT and BAT functionality promoting energy expenditure and the browning of epWAT (Carino et al. 2017b). Moreover, BAR501 has been affirmed as a promising lead compound in IBD. In murine models of colitis, BAR501 attenuates inflammation and immune dysfunction by shifting the polarization of colonic macrophages from the inflammatory phenotype M1 to the anti-inflammatory phenotype M2. The beneficial effects in these models are mediated by a GPBAR1-PKA-CREB-dependent mechanism with increased expression of IL-10 gene transcription in the intestine and enhanced secretion of IL-10 by macrophages (Biagioli et al. 2017).

Very recently, compounds **6**, **7**, and **8** have been identified as the first examples of lithocholic bile acid derivatives endowed with FXR agonism and GPBAR1 antagonism (Sepe et al. 2016b). In a proof of concept study affirming GPBAR1 a key factor in driving a migratory phenotype in gastric cancer cell lines, compound **7** inhibited the acquisition of a metastatic phenotype decreasing the expression of several genes associated with epithelial-mesenchymal transition in human gastric adenocarcinomas. This study affirms GPBAR1 antagonists as a novel pharmacological approach in the treatment of metastatic gastric cancers (Carino et al. 2016).

3.2 FXR Nonsteroidal Agonists (Fig. 5)

Steroidal FXR agonists suffered some limitations, such as poor aqueous solubility, poor bioavailability, and intense hepatic metabolization with unattractive pharmacokinetic profile. Moreover, several steroidal FXR agonists, as previously described, combined GPBAR1 agonistic properties, and consequently some GPBAR1-related side effects, such as pruritus.

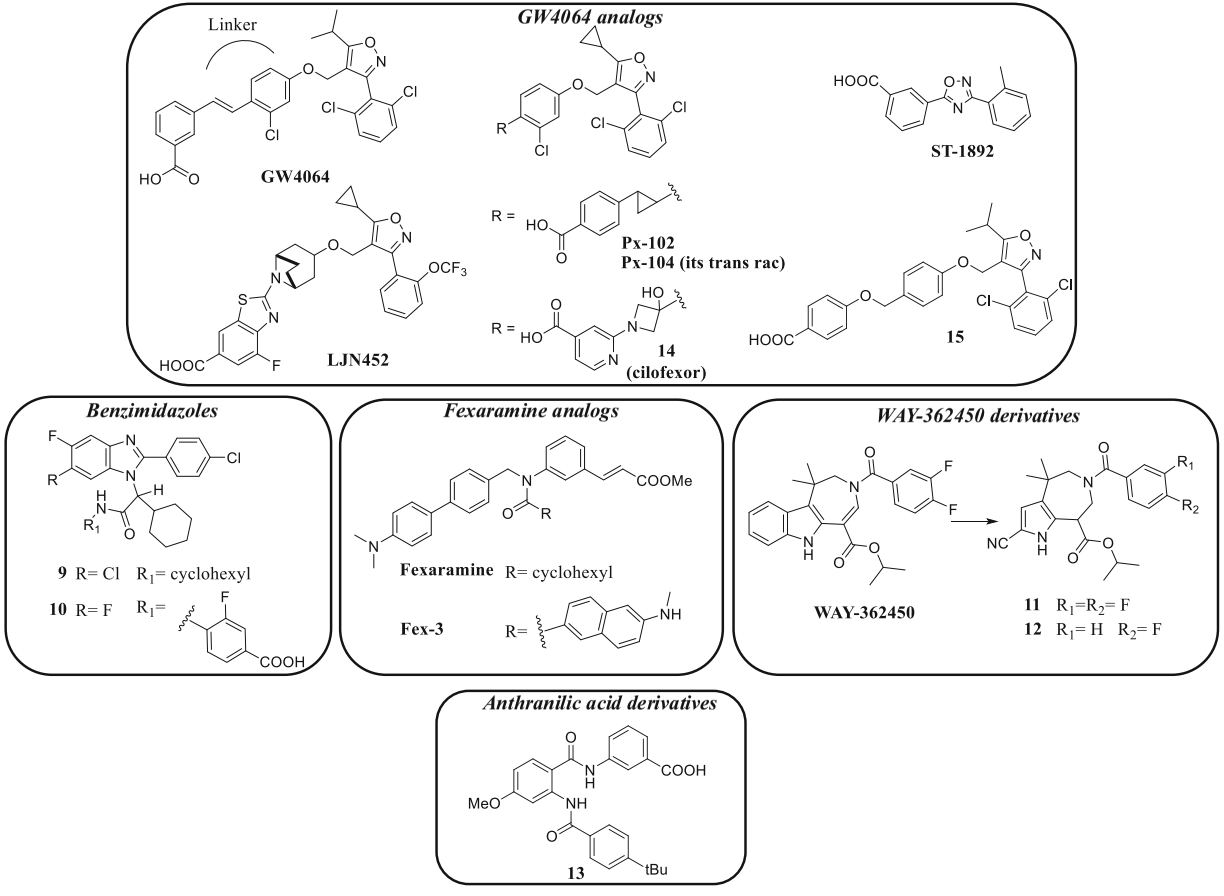


Fig. 5 FXR nonsteroidal agonists

For all these reasons, several pharmaceutical companies in recent years have been involved in the research of synthetic nonsteroidal FXR agonists, which could preserve the therapeutic potential and avoid adverse pharmacokinetic and pharmacodynamic properties. Altogether, few nonsteroidal FXR agonist chemotypes were been reported, including isoxazole derivatives (GW4064 analogs), benzimidazoles (compounds **9** and **10**) (Richter et al. 2011a, b; Sindhu and Srinivasan 2014), fexaramine analogs (fexaramine and Fex-3) (Downes et al. 2003; Zheng et al. 2017a; Wang et al. 2017b), and azepino[4,5-b]indole derivatives (WAY-362450 and its optimized derivatives **11** and **12**) (Flatt et al. 2009; Mehlmann et al. 2009). Recently, using the virtual screening techniques, a series of partial or full FXR agonists have been identified (Giancristofaro et al. 2018; Merk et al. 2014a, b; Deng et al. 2008). These compounds are anthranilic acid derivatives (Merk et al. 2014a, b); some of them endowed of high potency and efficacy as partial FXR agonists in a reporter gene assay (compound **13** $EC_{50} = 8 \pm 3$ nM) or pyrazolidine-3,5-dione derivatives (Deng et al. 2008).

Figure 5 summarizes the main scaffolds and the most recent identified compounds.

Among the above chemotypes, GW4064 represents a cornerstone in the development of nonsteroidal FXR agonists (Maloney et al. 2000). Even if endowed with high potency and efficacy, GW4064 clinical application was hampered by its hepatocellular toxicity, stilbene-mediated photo-instability, and poor pharmacokinetic properties.

Thus, in the last 10 years, several medicinal chemistry protocols have been focused in overcoming GW4064 drawbacks, with the identification of a large family of isoxazole derivatives, whose chemical features as well as the pharmacological properties have been widely discussed in some recent reviews (Crawley 2010; Sepe et al. 2015b; Xu 2016), and in the chapter "Nonsteroidal FXR Ligands: Current Status and Clinical Applications" by Gege et al. for this handbook. First, Px-102 (PX20606) and the corresponding trans-isomer Px-104 (Kinzel et al. 2016) show similar GW4064 FXR affinities but improved aqueous solubility and metabolic stability.

Px-102 has demonstrated beneficial effects in two animal models of prehepatic and intrahepatic portal hypertension reducing liver fibrosis, inhibiting sinusoidal remodeling, and improving hepatic vascular dysfunction (Schwabl et al. 2017).

Both molecules have recently progressed into Phase I clinical trial in NAFLD patients (Phenex Pharmaceuticals AG 2011, 2012, 2013).

On this trail, by introducing a hydroxyl-bearing four-membered ring as a linker between the middle and the terminal aryl rings, compound **14** (cilofexor), a potent FXR agonist with improved ADME profile, was identified (Kinzel et al. 2016). Compound **14** has reached the Phase II in NASH for evaluating the safety, tolerability, and efficacy (Gilead Sciences 2016).

Finally, the introduction of a bicyclic nortropine-substituted benzothiazole carboxylic acid moiety afforded LJN452 or tropifexor, able to activate FXR with an EC_{50} of 0.2 nM. LJN452 emerged as a new safe and well-tolerated drug, with improved pharmacokinetics parameters, such as low clearance and a longer terminal half-life (Tully et al. 2017). It has progressed into Phase II clinical trials for the

treatment of patients with PBC (Novartis Pharmaceuticals 2015) and NASH (Novartis Pharmaceuticals 2016).

Very recently, the fragmentation and structural optimization of GW4064 led to the identification of a new highly potent, soluble, and metabolically stable FXR agonist such as ST-1892 [EC_{50} (hFXR) = 7.2 ± 0.2 nM; aq. sol. = 33 mg/L (0.12 mM); metabolic stability: 54 ± 1% after 60 min] (Flesch et al. 2015) and compound **15** (Sepe et al. 2019) (EC_{50} = 0.30 μM), with this latter demonstrating a promising candidate in protecting mice from acute liver toxicity caused by APAP misuse.

3.3 FXR Antagonists (Fig. 6)

As pointed before, large research interest has been devoted at the identification and development of FXR agonists. Indeed, several side effects emerged, and recently the unexplored field of FXR antagonism has been targeted by academia and several companies.

Although the full therapeutic potential of this approach remains to be clarified, FXR antagonists have been proved beneficial in animal model of cholestasis and hypercholesterolemia as well as in pancreatic and colon cancers.

So far, few chemical classes of FXR antagonists have been reported, including compound **16**, a thienyl containing GW4064 analogue (Huang et al. 2015), pyrazole-4-carboxamides (Yu et al. 2014) such as compound **17**, 1,3,4-trisubstituted-pyrazolones (e.g., **18**) (Huang et al. 2012), and benzimidazoles (e.g., **19**) (Teno et al. 2018).

Finally, different series of moderate FXR antagonists have been discovered via high-throughput screening approach such as hydroxyacetophenone (PHA) derivatives, including compound **20** which proved to be the most potent in this series (Liu et al. 2014b) or 3-(tert-butyl)-4-hydroxyphenyl 2,4-dichlorobenzoate (**21**) and its 2,6-dichloro-4-amidophenyl derivative NBD, showing improvement in stability and potency (Song et al. 2015; Xu et al. 2015).

Very recently, SIPI-7623, a structurally related compound of PHA (Deng et al. 2018) and a new class of compounds, characterized by a *N*-phenylbenzamide scaffold (Schmidt et al. 2018), were identified as full FXR antagonists. Systematic optimization on the above *N*-phenylbenzamide scaffold afforded the most potent FXR antagonist published so far, compound **22**, which showed low nanomolar potency and selectivity, low cytotoxicity, and high metabolic stability (Schmidt et al. 2018).

Finally, a new chemotype of FXR antagonists featuring a naphthyl at C-3 and different protonable *N*-bearing heterocyclic substituents at C-5 on a central oxadiazole core has been reported by our research group in a very recent time (Festa et al. 2019). Compound **23**, the most potent and selective FXR antagonist of this series, showed also excellent pharmacokinetic properties.

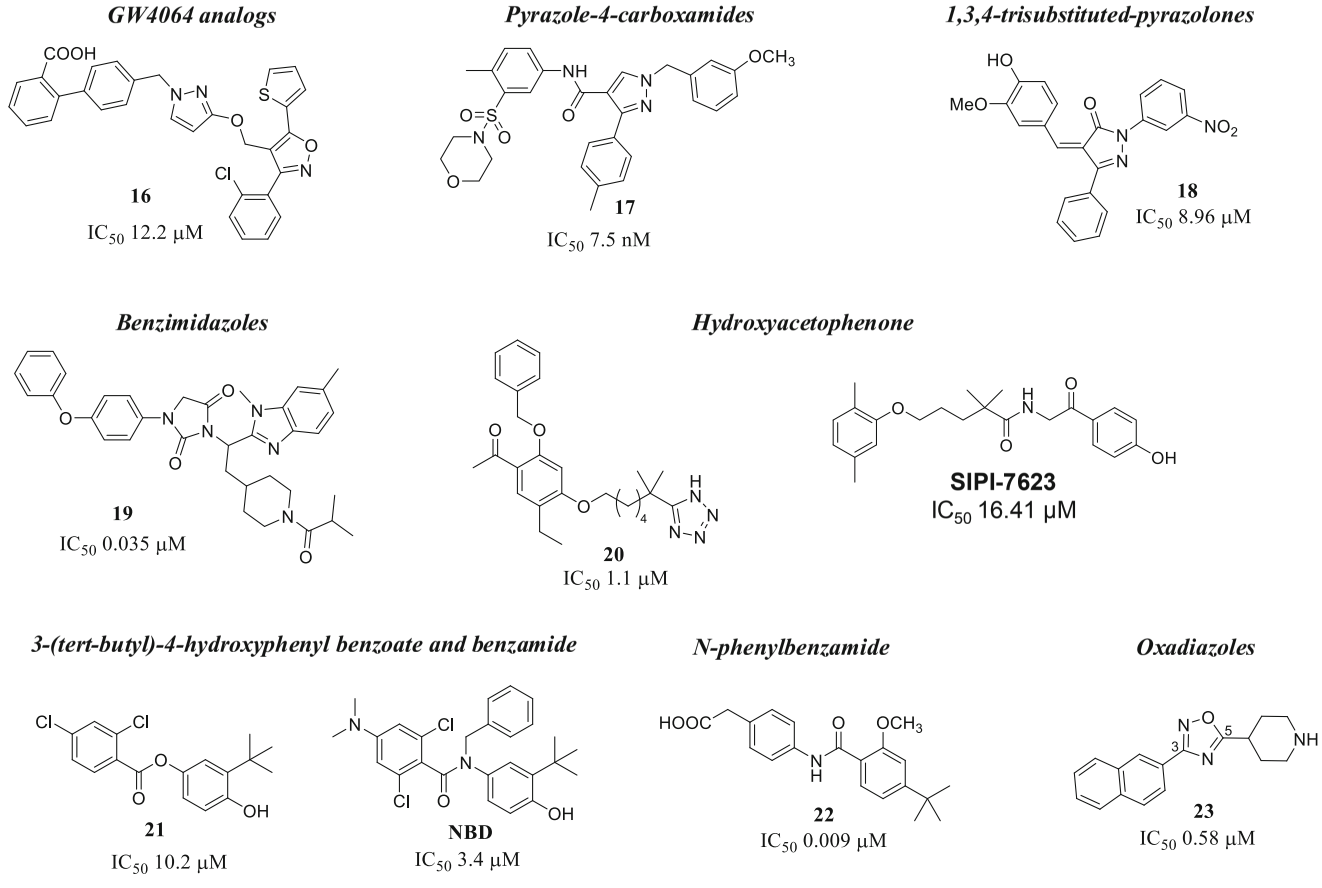


Fig. 6 FXR nonsteroidal antagonists

3.4 GPBAR1 Nonsteroidal Agonists (Figs. 7 and 8)

In order to obtain higher selectivity over other bile acid-mediated pathways, several examples of nonsteroidal chemical templates have been reported as GPBAR1 selective agonists (Budzik et al. 2010; Evans et al. 2009; Herbert et al. 2010) by various pharmaceutical companies and research groups. The chemical classes so far identified are reported in Fig. 7 and could be summarized as follows:

- 3-Aryl-4-isoxazolecarboxamides (compound **24**)
- 3-Aminomethylquinolines (compounds **25a** and **25b**)
- 2-Phenoxynicotinamides (compounds **26a** and **26b**)
- 4-Phenylpyridines and pyrimidines (compounds **27a** and **27b**)
- 3,4,5-Trisubstituted 4,5-dihydro-1,2,4-oxadiazoles (compounds **28a** and **28b**)
- Nipecotamide derivatives (compound **29**)
- Oximes (compound **30a** and **30b**)
- Diazepine (**SB-756050**)

All these compounds have been previously described by Xu in a recent and exhaustive review (Xu 2016). Of interest, among the above molecules, **SB-756050** has reached the Phase I clinical trials for the treatment of type 2 diabetes mellitus (Hodge et al. 2013), but complex and inconsistent pharmacodynamic effects emerged.

Starting from this background, two different routes have been followed:

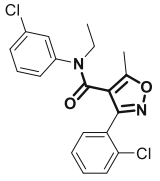
- Optimization of pharmacokinetic and pharmacodynamic parameters in vivo
- Maximization of gut-restricted activity

Most of the reported GPBAR1 agonists possess insufficient potency and/or lack metabolic stability. The optimization of the pharmacokinetic and pharmacodynamic parameters could increase the possibility of discovering new tools with better in vivo potency and low side effects. A new series of imidazoles (compound **31**) (Lasalle et al. 2017) and tetrahydrobenzimidazoles (compound **32**) (Zhang et al. 2017) have been disclosed as orally efficacious GPBAR1 agonists, showing sub-micromolar EC_{50} values for mGPBAR1 and hGPBAR1.

Recently, compound **33**, a 2-thio-imidazole derivative, was identified as a novel, potent, and selective GPBAR1 agonist (EC_{50} = 57 pM) (Agarwal et al. 2016) endowed with favorable pharmacokinetic profile in vivo and potent glucose lowering effects in DIO C57 mice, with an ED_{50} of 7.9 mg/kg and ED_{90} of 29.2 mg/kg.

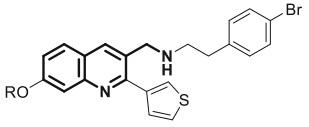
Otherwise, in order to maximize gut restriction and optimize pharmacokinetic and pharmacodynamic parameters in vivo, 4-phenoxynicotinamide and 4-phenoxypyrimidine-5-carboxamide scaffolds have been elaborated. Introduction of a mini-PEG linker, a quaternary ammonium function, or polar surface area such as a D-glucamide affording compounds **34** (Duan et al. 2015), **OL3** (Ma et al. 2016), **35** (Cao et al. 2016), and a new thiazolidine chemotype **36** (Chen et al. 2018), as gut-restricted agonists. Gut-restricted agonists have the advantage to minimize

3-aryl-4-isoxazolecarboxamides



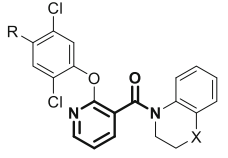
24
pEC_{50} 5.3

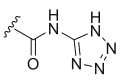
3-aminomethylquinolines



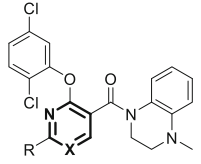
25a R=Me EC_{50} 65 nM
25b R=H EC_{50} 5.1 μM

2-phenoxynicotinamides



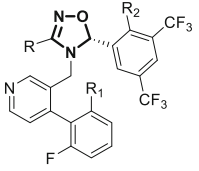
26a X=CH_2 R=H EC_{50} 10 nM
26b X=  R=  EC_{50} 76 nM

4-phenylpyridines/pyrimidines



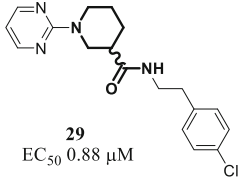
27a X=CH R=H EC_{50} 6.1 nM
27b X=N R=OH EC_{50} 3.9 nM

3,4,5-trisubstituted 4,5-dihydro-1,2,4-oxadiazoles



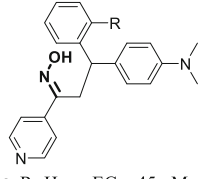
28a R=Ph R_1=H R_2=H EC_{50} 79 nM
28b R=2-furyl R_1=F R_2=Cl EC_{50} 1.4 nM

Nipecotamide



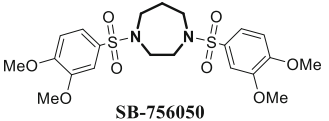
29
EC_{50} 0.88 μM

Oximes



30a R=H EC_{50} 45 nM
30b R=Me EC_{50} 12 nM

Diazepine



SB-756050
EC_{50} 1.3 μM

Fig. 7 GPBAR1 nonsteroidal agonists

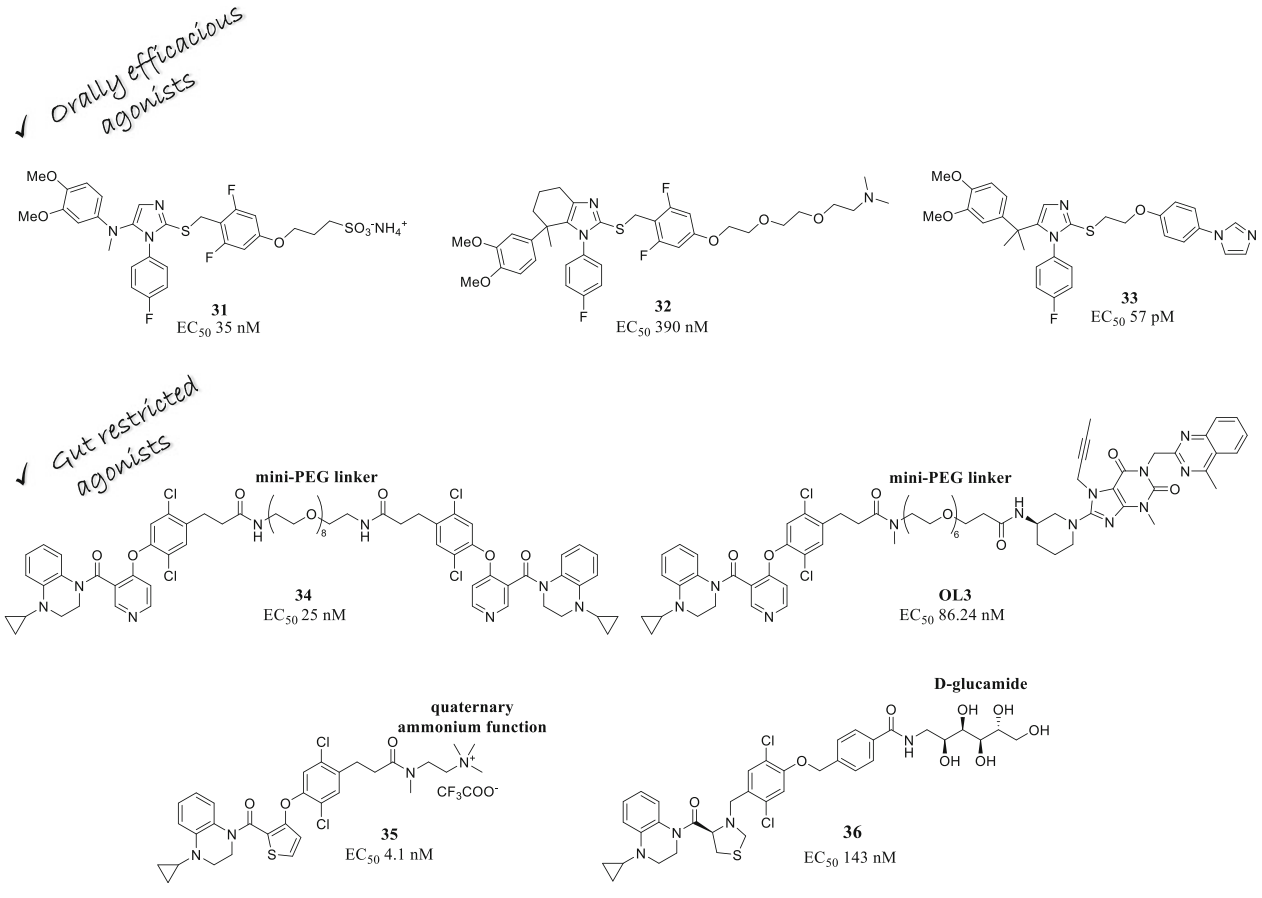


Fig. 8 New generation of GPBAR1 nonsteroidal agonists

undesirable side effects, such as excessive gallbladder filling, blockade of gallbladder emptying, itching, and cardiovascular issues associated with systemic GPBAR1 agonism.

4 Conclusion

As outlined before the first examples of FXR and GPBAR1 modulators were identified from the fascinating world of natural products remaining the principal source of active secondary metabolites that highly contributed to the total amount of FDA-approved agents.

Natural compounds represent privileged scaffolds with unusual structural features, biochemical specificity, and other molecular properties. However, their clinical use is limited by the difficulty to obtain sufficient amounts of compound, unknown biological targets and mechanism of action, and a poor pharmacokinetic profile. For this reason, the interest of today's research has been shifted toward an alternative and rationale development of new molecules, including high-throughput screening, virtual screening, and total synthesis of small molecules.

Starting from the structures of bile acids as endogenous regulators of FXR and GPBAR1 functions, two different research directions have been followed in the identification of steroidal and nonsteroidal modulators. In this review we have reported the most important leads identified, some of them currently in advanced clinical trials.

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Nonsteroidal FXR Ligands: Current Status and Clinical Applications

Christian Gege, Eva Hambruch, Nina Hambruch, Olaf Kinzel, and Claus Kremoser

Contents

Abstract

FXR agonists have demonstrated very promising clinical results in the treatment of liver disorders such as primary biliary cirrhosis (PBC), primary sclerosing cholangitis (PSC), and nonalcoholic steatohepatitis (NASH). NASH, in particular, is one of the last uncharted white territories in the pharma landscape, and there is a huge medical need and a large potential pharmaceutical market for a NASH pharmacotherapy. Clinical efficacy superior to most other treatment options was shown by FXR agonists such as obeticholic acid (OCA) as they improved various metabolic features including liver steatosis as well as liver inflammation and fibrosis. But OCA's clinical success comes with some major liabilities such as pruritus, high-density lipoprotein cholesterol (HDL_c) lowering, low-density lipoprotein cholesterol (LDL_c) increase, and a potential for drug-induced liver toxicity. Some of these effects can be attributed to on-target effects exerted by FXR,

C. Gege · E. Hambruch · N. Hambruch · O. Kinzel · C. Kremoser (✉)
Phenex Pharmaceuticals AG, Drug Discovery Research, Heidelberg, Germany
e-mail: claus.kremoser@phenex-pharma.com

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but with others it is not clear whether it is FXR- or OCA-related. Therefore a quest for novel, proprietary FXR agonists is ongoing with the aim to increase FXR potency and selectivity over other proteins and to overcome at least some of the OCA-associated clinical side effects through an improved pharmacology. In this chapter we will discuss the historical and ongoing efforts in the identification and development of nonsteroidal, which largely means non-bile acid-type, FXR agonists for clinical use.

Keywords
Cilofexor · Farnesoid X receptor · GS-9674 · NASH · Tropifexor

1 Overview and Brief History of FXR Drug Discovery

Soon after the identification of bile acids to be likely the prevailing endogenous ligands of FXR (Makishima et al. 1999; Parks et al. 1999), the nuclear receptor drug discovery group around Timothy Willson at GlaxoSmithKline research site at Research Triangle Park, North Carolina, initiated a screen against a synthetic compound library derived from combinatorial chemistry (Maloney et al. 2000). This screen led to the identification of GW4064 (Fig. 3), the first synthetic FXR agonist with decent potency (30 nM in a cell-based assay) and selectivity against other nuclear receptors. In the first publication by this group, GW4064 was tested in Fisher rats to yield significant lowering of serum cholesterol and triglycerides in this animal model. Several publications that were released in the early 2000s and that mainly employed 6-ethyl-CDCA (now known as INT-747 or OCA) as a tool FXR agonist demonstrated strong hepatoprotective effects of FXR activation in various animal models of liver cholestasis and fibrosis (Fiorucci et al. 2004, 2005b; Pellicciari et al. 2002). This was the basis for the early Phase II trials of INT-747/OCA in PBC which is the most severe autoimmune-type liver disorder that presents with such sequelae. In 2005 and 2006, three independent publications were released where the respective authors showed that activation of FXR leads to beneficial insulin-sensitizing, glucose-, lipid-, and cholesterol-lowering effects in mice (Cariou et al. 2006; Stayrook et al. 2005; Zhang et al. 2006), thus implicitly suggesting a FXR agonist-based treatment for metabolic diseases such as type 2 diabetes (T2D). This spurred the efforts in the biopharmaceutical industry to find novel, improved synthetic FXR agonists with appropriate drug-like properties, and in the following 5 years, companies such as GlaxoSmithKline, Eli Lilly, F. Hoffmann-La Roche, Wyeth (now Pfizer), and Phenex Pharmaceuticals patented several novel derivatives of GW4064 (the so-called "hammerhead" class; see Fig. 2 and Gege et al. 2014) but also some novel chemotypes such as WAY-450. The reason why many major pharmaceutical companies have intensely worked on the medicinal chemistry of synthetic FXR agonists but failed to test them in human patients was likely that the pure antidiabetic effects of FXR agonists were limited in their efficacy and the lipid-lowering effects typically came with the liability that it was accompanied by HDL_c lowering in most preclinical species tested including mice and monkeys (Evans et al.

2009; Hambruch et al. 2012; Hartman et al. 2009). Thus, a FXR agonist, just based on the preclinical findings, would not represent the ideal prototype of a novel antidiabetic or lipid-lowering drug.

On Jan 8, 2014, an absolute turning point in the drug discovery history of FXR agonists was reached: Intercept Pharmaceuticals released topline data from their FLINT trial, a Phase IIb study investigating OCA in a liver indication previously only known to hepatologists named nonalcoholic steatohepatitis or in brief NASH (Neuschwander-Tetri et al. 2015; Press Release 2014). OCA clearly improved the NASH liver histopathology in all relevant categories even in an interim analysis when roughly only a half of the 280 planned for patients had been analyzed. The stock price of Intercept skyrocketed on that day from 72 to 275 US$ which indicated the enormous market potential that was projected for NASH. We will discuss the application of FXR agonists in NASH later, but this single event boosted the commercial as well as academic drug discovery efforts enormously.

Companies with a declared focus on liver diseases such as Gilead Sciences, Novartis, and Allergan increased their efforts in finding appropriate FXR agonist drug candidates. These efforts gave rise to LJN452 aka tropifexor which is a very potent synthetic GW4064 derivative stemming from Novartis internal drug discovery (Tully et al. 2017) and to GS-9674 aka cilofexor (see Fig. 1 for structure of both compounds) which was originally made and patented by Phenex Pharmaceuticals but then sold to Gilead Sciences in a 470 M US$ transaction. These two compounds are clinically the two most advanced synthetic FXR agonists, followed by a second FXR agonist from Novartis, LMB763 aka nidufexor, which has a completely different structural motif compared to the isoxazole-type tropifexor and cilofexor. Allergan has licensed a compound from Akarna which is likely a derivative of the WAY-450 (Fig. 1) but has not officially initiated clinical development of this drug, probably because Allergan has partnered with Novartis, thereby gaining access to their two advanced FXR agonists.

Although most companies that have FXR agonists in clinical development run trials in PBC and PSC, NASH is clearly the most appealing indication given its enormous market potential (see Sect. 5). Twenty years after its discovery as a bile acid receptor and an important regulator of liver protection and intermediary metabolism, FXR has been torn out of the academic atmosphere into the limelight of pharmaceutical companies and investors' interests. Still, there are several open questions, in particular with regard to the known and well-documented side effects of FXR agonist-based therapies, such as:

- Are the signs of pruritus that are now documented not only for OCA but also for synthetic nonsteroidal FXR agonists really a direct outcome of FXR activation? Is it common to all FXR agonists, or are there differences depending on the chemical nature (steroidal versus nonsteroidal)?
- Given that for FXR activation beneficial effects on steatosis, inflammation, fibrotization, and liver endothelial recovery are published: Will a pure FXR agonist-based therapy suffice for NASH, in particular?

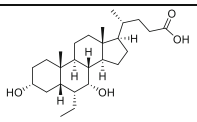
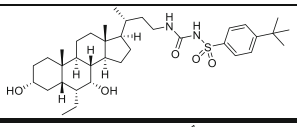
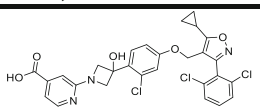
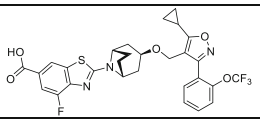
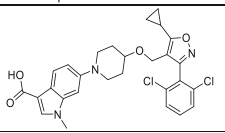
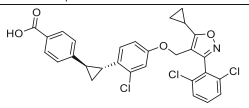
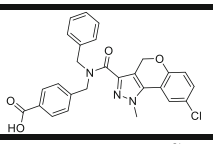
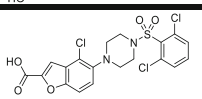
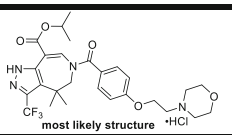
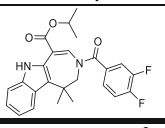
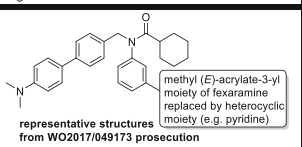
Name	Alternative Names	Structure	Originator	Clinical Status
OCA	obeticholic acid INT-747 6-ethyl-CDCA		University of Perugia Intercept Pharma.	Approved for PBC as Ocaliva® Phase III for NASH and PSC
EDP-305			Enanta	Phase Ia/b finished
cilofexor	GS-9674 Px-201		Gilead Sciences (acquired from Phenex)	Phase IIb in NASH Phase II in PBC, PSC
tropifexor	LJN452		Novartis	Phase II in NASH
TERN-101	LY2562175		Lilly (licenced to Terns Pharmac. Inc. in 2018)	Phase I finished
Px-102 (racemate) Px-104 (enantiomer)	PX20606 (research code)		Phenex Pharmaceuticals	Phase IIa in NAFLD (abandoned)
nidufexor	LMB763		Novartis	Phase II in NASH
EYP001	PXL007		Enyo Pharma (licensed from Poxel)	Phase II in NASH and Hepatitis B infection
AGN-242266	AKN-083		Allergan (acquired through Akarna Therapeutics)	Phase I
WAY-450	WAY-362450 XL-335		Wyeth (now Pfizer, acquired from Exelixis/ X-Ceptor)	Phase I (abandoned)
MET409			Metacrine	Phase Ia

Fig. 1 FXR agonists in clinical development

- Are there any means to dial in certain desired effects into and undesired effects out of FXR agonists? If so, what could be the rationale behind such efforts?

2 Structural Diversity of Nonsteroidal FXR Agonists

Figure 1 lists all published FXR agonists that have reached Phase I human clinical testing at least. Two of them, WAY-450 and Px-102/104, have been abandoned for undisclosed reasons, but the remaining eight ones are in active clinical trials. Among these substances two (OCA and EDP-305) have a steroid scaffold, whereas the other ones are fully synthetic nonsteroidal structures. Figure 2 represents a kind of "evolutionary tree" of nonsteroidal FXR structures. What can be nicely observed is that the release of positive Phase IIb "FLINT" trial data from OCA in NASH patients in January 2014 has triggered a burst of synthetic and – with a due delay of 2–3 years – also of patenting activities of novel synthetic FXR agonists.

The richest class of nonsteroidal FXR agonists is the so-called "hammerhead" class of trisubstituted isoxazole core compounds pioneered by the aforementioned GW4064. GW4064 could serve only as a tool compound since its bioavailability and its in vivo half-life were very limited. Beyond its pharmacokinetic (PK) limitations, the chemical structure of GW4064 harbors a stilbene moiety that could appear as a photolabile and potential metabolite soft spot. Such stilbene moieties are known to potentially demonstrate potent estrogenic effects, and indeed it was found that GW4064 changes muscle metabolism by acting on estrogen-related receptor alpha (ERRα) independent from its activity on FXR (Dwivedi et al. 2011). Moreover, GW4064 was also found to inhibit breast and Leydig tumor cell growth by inhibiting aromatase expression which is another indication of estrogen-like activity of GW4064 (Swales et al. 2006). Various attempts mainly by GlaxoSmithKline, Lilly, and Phenex have been undertaken to replace the photolabile and potentially pro-estrogenic stilbene moiety of GW4064 (reviewed in Gege et al. 2014) by at least equipotent linkers to yield more stable and drug-like compounds. The first to reach Phase I testing was Px-102 from Phenex where the double bond linker was replaced by a cyclopropyl moiety. In this constellation, potentially four stereoisomers, the *cis*- and *trans*-substituted cyclopropyls and their enantiomers, are possible, but the *cis* isomer can be ruled out for the energetically unfavored sterical conformation that this isomer would induce. Phenex tested the racemic version, Px-102 (also published as PX20606 in scientific literature), in two human Phase I studies in healthy volunteers first during 2011 and 2012 (NCT01998672, NCT01998659) and switched to the eutomer, Px-104, for a small exploratory Phase II study (NCT01999101) after which further development was abandoned for undisclosed reasons. In a subsequent publication, Kinzel et al. laid out their intention to replace the hydrophobic and stereocenter-containing cyclopropyl with four-membered ring systems and to introduce more polarity into the hydrophobic aromatic ring systems (Kinzel et al. 2016). This was achieved by replacing the terminal COOH-bearing aryl with a heteroaryl such as pyridine by using the four-membered N-containing azetidinyl as the middle linker element and by further adding a hydroxy group to this

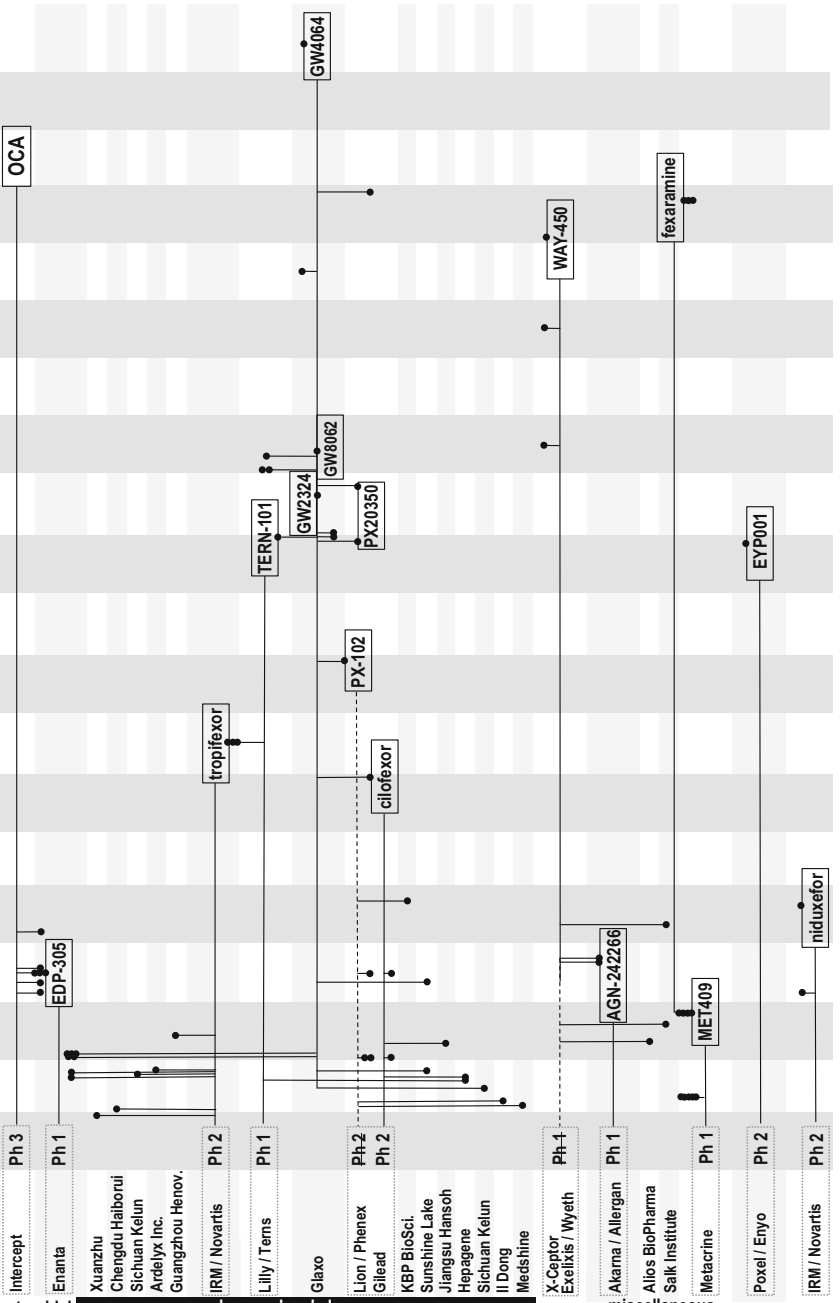


Fig. 2 "Evolutionary tree" of FXR agonist structures that reached clinical trial stage (clustered according to structural similarity)

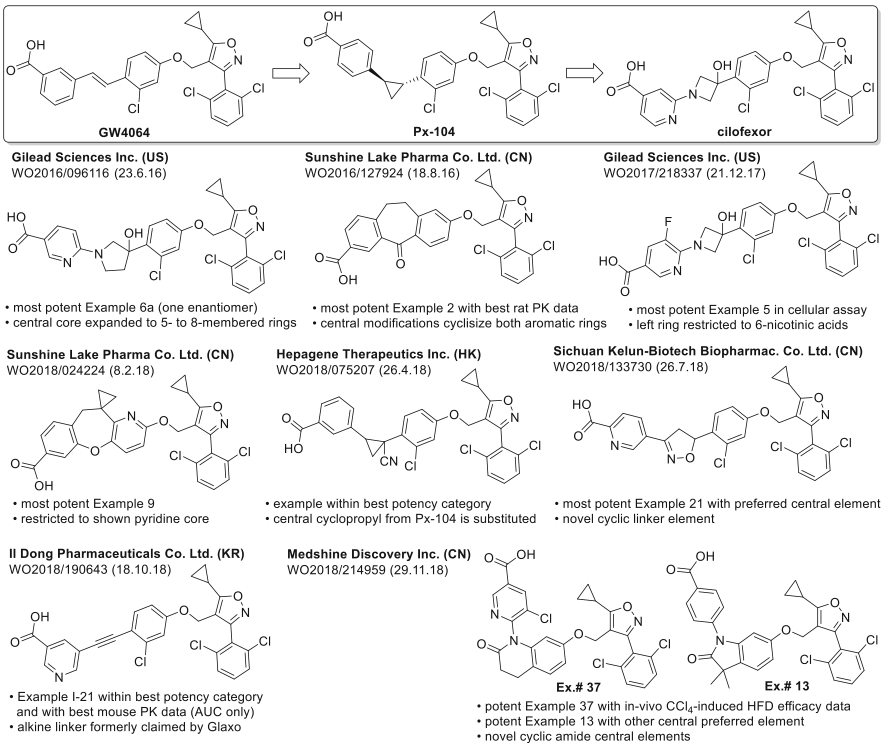


Fig. 3 From GW4064 to cilofexor and follow-on patent applications

cyclic system which is said to engage into a polar interaction and also to increase hydrophilicity. These improvements are best exemplified in the clinical development candidate GS-9674 which was acquired by Gilead Sciences and is now officially termed "cilofexor" (Fig. 3).

From this "early" period of FXR synthetic activity from 1999 till 2014, only cilofexor and the Lilly compound LY2562175 (Genin et al. 2015) survived as clinical candidates. LY2562175 was tested in a European Phase I study and subsequently licensed to Terns Pharmaceuticals and renamed into TERN-101. The company plans to test it in a Phase II study in NASH patients in China. The novelty of this hammerhead isoxazole compound is that the aromatic ring attached to the oxymethylene linker of the isoxazole is replaced by a saturated piperidine ring and the carboxylic acid-bearing terminal part is embodied as a bicyclic ring (Fig. 4).

Building on this core motif of the Lilly compound, researchers at Novartis have modified the middle saturated piperidine by adding an ethylene bridge to form an 8-azabicyclo[3.2.1]octane system and by replacing the indole with a reverse benzothiazole. This yielded a single-digit nanomolar potent and fully efficacious FXR agonist with high in vivo efficacy called LJN452, now tropifexor, and is one of two Novartis compounds in Phase II testing in NASH patients and other indications. Several, mainly Asian biotech companies filed me-too patent applications, which exploit presumed free chemical space (Fig. 4). For HNC143 and HNC180, the X-ray

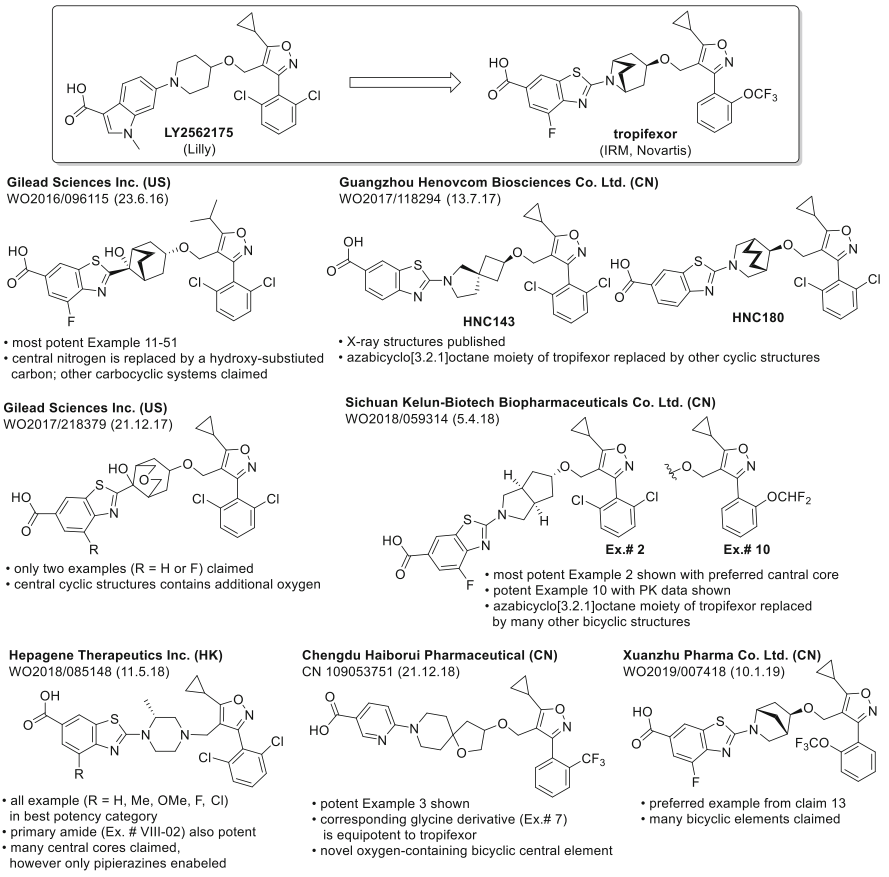


Fig. 4 LY2562175 as another embodiment of hammerhead isoxazoles giving rise to tropifexor and many follow-on patent application series

structure bound to FXR was published (Wang et al. 2018). Novartis' nidufexor is a completely novel chemotype, and only sparse data from a Phase I study with this drug candidate has been released which shows that it has medium potency but also displays HDL_c-lowering effects (Huttner et al. 2017).

The replacement of the isoxazole moiety was already extensively investigated (reviewed in Gege et al. 2014) – 1,2,3-triazole or pyrazole first described by Lilly remained promising replacements for the isoxazole. Isothiazole was previously not covered, and two Asian companies recently claimed these structures (Fig. 5): KBP Bio Sciences focus on a me-too of an old Phenex application from 2009, while Jiangsu Hansoh Pharmaceuticals focus on a Phenex application covering cilofexor. Additional patent space is intended with isoxazoles by omitting the hydroxy element in the azetidine linker.

Enanta has taken a more universal approach to generate own intellectual property (Fig. 6): They use carboxylic acid bioisosteres to replace the terminal carboxylic acids of known hammerhead-type structures, thereby yielding compounds that are

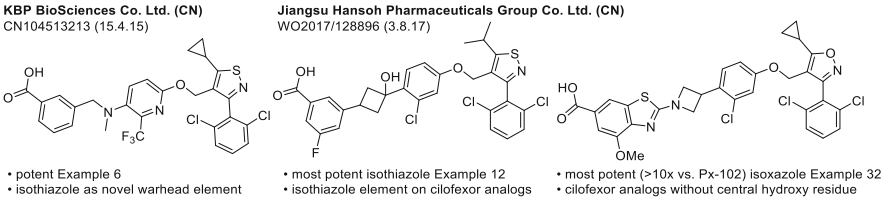


Fig. 5 Recent hammerhead modifications by replacing the isoxazole motif by an isothiazole moiety

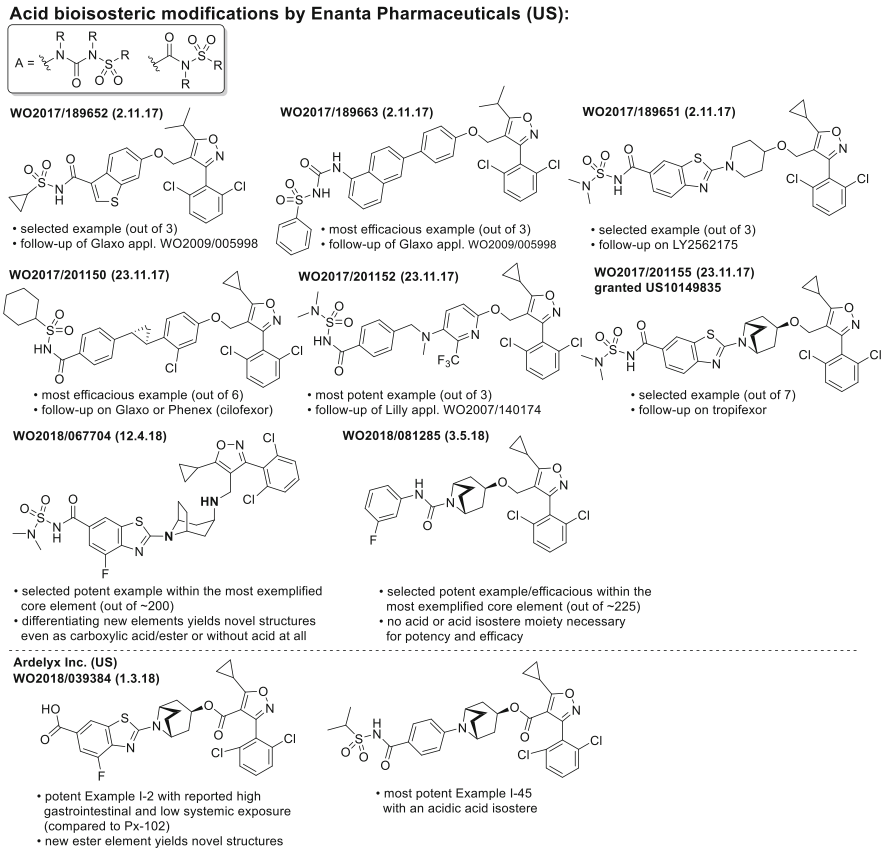


Fig. 6 The Enanta strategy to claim acid isosteres but also other novel variations of the isoxazole motif

about equipotent to their carboxylic acid counterparts. The acid is involved in hydrogen bond interactions as well as in ionic interactions with arginines at this part of the ligand-binding domain (LBD), so it is possible to replace a normal carboxylic acid with a bioisostere. Enanta uses alkyl- or aryl-substituted sulfonyl-acyl amides with an acidic N-H. The aryl/alkyl substituent will presumably protrude

out of the ligand-binding pocket into the water environment. This might explain why many small alkyl/aryl substituents are allowed for. It has to be kept in mind that this type of acid isostere unlike a real carboxylic acid cannot be taurine- or glycine-conjugated in vivo, an important feature that we need to consider when discussing their PK behavior (see below). The newer Enanta patent applications, however, take a different approach by replacing the *O*-methylene effectively with a *N*-methylene preferably on Novartis' 8-azabicyclo[3.2.1]octane scaffold (WO2018/067704). The other new application introduces a urea system between the middle azabicyclo system and the terminal aryl ring. Here, even the terminal carboxylic acid or acid isostere is omitted (WO2018/081285) still furnishing FXR agonists within the best potency range (<10 nM). Ardelyx follows Enanta and Novartis, then, using their azabicyclo ring replacement in combination with the Novartis or with Enanta headgroups but with an ester linker instead of an *O*-methylene linker (WO2018/039384).

Another family of structures and patents with decent FXR agonist activity was built around the WAY-450, originally discovered at X-Ceptor Therapeutics (then called XL-335) and then acquired by Exelixis and finally by Wyeth. For WAY-450 several animal studies showed its significant potential in various applications (Evans et al. 2009; Flatt et al. 2009; Hartman et al. 2009; Zhang et al. 2009). WAY-450 suffered from insufficient aqueous solubility since it does not have a free ionizable group and attempts were undertaken to improve this shortcoming by introducing more polar elements and a terminal morpholine-based solubility handle (Lundquist et al. 2010). Akarna Therapeutics was the first "follower" to take WAY-450 as a template to come up with own improved but still related structures (Fig. 7), and Alios BioPharma is another company fishing in the same water as Akarna with a series of WAY-450 replacements. The Salk Institute has also filed a patent application on deuterated WAY-450 structures. However how these deuterated compounds differ from their normal hydrogen-decorated counterparts is not exemplified in the patent application nor published in a scientific journal.

Fexaramine (Fig. 8) is one of the earliest synthetic nonsteroidal FXR agonists that was discovered in the groups of Kyriacos C. Nicolaou and Ron Evans (Downes et al. 2003). Although it shows double-digit nM potency at FXR in in vitro assays, it has some features that prevent it from being drug-like such as an overall hydrophobicity and thus insolubility, two aniline substructures with a potential risk of mutagenicity (Ames et al. 1975), and the terminal aryl-acrylic acid ester which can act as a potential Michael acceptor (Amslinger 2010). However, fexaramine has become the prototype of an intestinally restricted FXR agonists (Fang et al. 2015) and has demonstrated to elicit potent beneficial metabolic effects (see discussion at the end of this section) while avoiding the side effects that come with liver FXR activation. Metacrine was set up as a commercial company to explore the potential of fexaramine further, and a key step in improving fexaramine was the replacement of the acrylate ester by various other moieties including aryl-aryl systems, aromatic acid amides, and oxy- or amino-methylene linker elements (with WO2017/049173 considered the most important one, since prosecuted in many countries). It is likely that Metacrine's MET409, their clinical candidate which is currently in Phase I

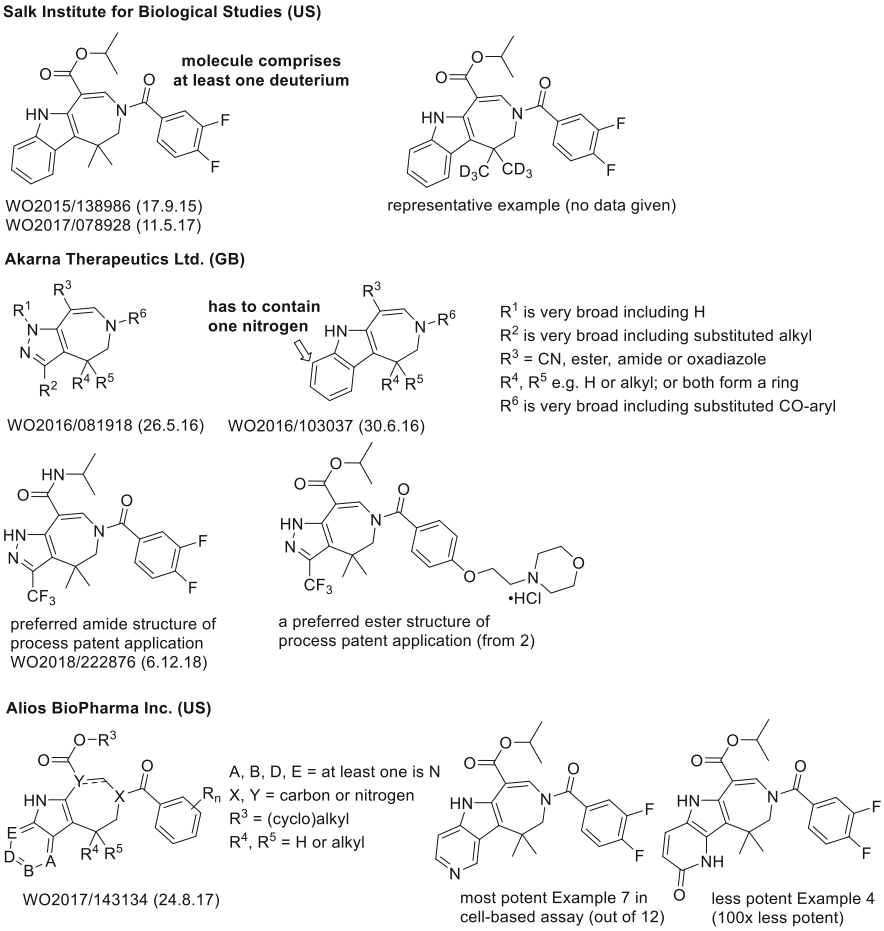


Fig. 7 The series of WAY-450 and its follow-on derivatives

clinical testing, is based on a structure covered by this patent application although Metacrine has disclosed in 2018 five further international patent applications with further improvements on other areas of the molecule (e.g., saturate the inner aryl of the biphenyl moiety or substitute the cyclohexyl moiety).

Further independent FXR structures are summarized in Fig. 8. Noteworthy is the identification of MFA-1 as a potent FXR agonist (Soisson et al. 2008). Although MFA-1 has a steroidal structure, it binds to FXR very differently from bile acids in that it is appr. 180° rotated as was demonstrated by X-ray analysis of a FXR-MFA-1 cocrystal structure. As early as 2003, another set of FXR agonists was disclosed in a patent application by a team from Lion Bioscience which later appeared as founders of Phenex Pharmaceuticals (WO2003/016280; WO2003/015777; WO2003/016288). These patent applications cover structures centered around screening hit TR0800012996 that was described with a potency of 0.5 μM in a cellular reporter assay. This compound contains a central, trisubstituted 2-aminopyridine moiety, and

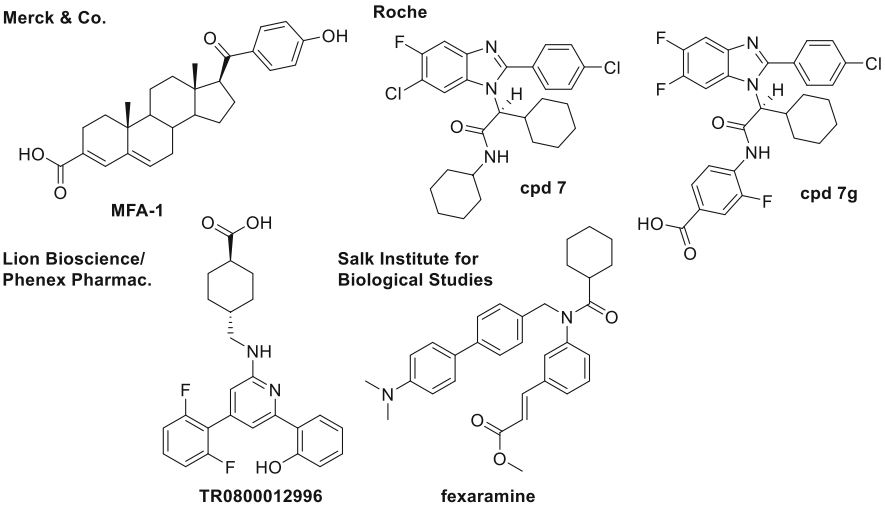


Fig. 8 Miscellaneous FXR agonists: MFA-1, a potent steroidal FXR agonist, yet with a reverted binding mode; TR080012996, an initial FXR screening hit identified at Lion Bioscience/Phenex; Cpd 7 and 7g prototypic benzimidazoles from Roche; fexaramine from the Salk Institute for Biological Studies

compounds with the same core structure are claimed in WO2003/016280. Replacement of the central 2-aminopyridine with 2-aminopyrimidines or with 2-aminothiazoles yielded compounds of similar potency, but none of them was clinically developed. In 2011, a team from Roche published series of substituted benzimidazole FXR agonists (Richter et al. 2011a, b). They were later shown to elicit certain lipid-lowering effects but appeared to be partial FXR agonists with limited in vivo potency compared to isoxazole-hammerhead-type FXR agonists (Gardès et al. 2013).

Worth mentioning is also the Poxel/Enyo compound EYP001 since this molecule is in Phase II testing for hepatitis B infection (HBV) as well as for NASH treatment. Unfortunately, there is no peer-reviewed publication on this compound, and the patent applications are not very informative with regard to its pharmacological properties.

In summary, the drug discovery industry has undertaken serious efforts to identify, optimize, and finally develop nonsteroidal FXR agonists all the way from initial hit identification up to late-stage clinical trials. The one series that is really outstanding are the hammerhead-type isoxazole core structure compounds, pioneered and first exemplified by GW4064. This chemical motif has the advantage of providing decent potency along with FXR selectivity due to its unique binding mode (see detailed discussion in Gege et al. 2014). The fact that this series has given rise to at least four different clinical candidates (Px-102, cilofexor, tropifexor, TERN-101) and that several mainly Asian followers try to copy this motif in own patent applications shows that this type of FXR agonist seems to have unique and superior properties. However, it needs further detailed investigation to uncover what exactly makes this particular structural motif so favorable.

Nevertheless, there are several other compounds of independent structures in clinical development, and it remains to be shown which ones will ultimately arrive in mankind's pharmacopeia.

3 Pharmacology of Nonsteroidal FXR Agonists

In this section we provide an overview on assay formats including considerations on easiness of setup, information content and limitation, as well as meaningfulness and positioning in the drug discovery cascade.

A screen or a medicinal chemistry effort starts with the desired properties of the target molecule and with the assay setup. There are various standard formats for nuclear receptor assays, and since there are many reference agonists for FXR commercially available, there are no special efforts or requirements necessary to launch a screening campaign for FXR agonists. The FXR agonists currently in clinical development all have potencies of $\leq$100 nM in typical biochemical assays – a potency in the low double-digit or even single-digit nanomolar range is probably desirable. There are various modalities for FXR agonists that need to be considered (partial or selective agonism, transporter dependency, tissue selectivity, etc.) but this will be discussed in subsequent sections.

In general, nuclear receptors such as FXR bind to their cognate response genes promoters as heterodimers with retinoid X receptor (RXR), the universal permissive heterodimer partner for type 2 nuclear receptors such as FXR, liver X receptors (LXRs), peroxisome proliferator-activated receptors (PPARs), vitamin D receptor, or pregnane X receptor (PXR). All nuclear receptors undergo a conformational rearrangement upon ligand binding in that the C-terminal Helix 12 which is located at the outside surface of the ligand-binding domain is "locked" into the active conformation through appropriate interactions with the agonist (Brzozowski et al. 1997; Gronemeyer et al. 2004). The active conformation is the conformation that allows for so-called coactivator proteins to bind to the RXR-FXR heterodimer and adapt it to other chromatin remodeling factors that are necessary to open the chromatin around the transcriptional start site for the initiation of transcription. This is a very complex event that involves up to 50 different proteins in a certain temporal and spatial order which is beyond the scope of this book chapter to be described in all details but reviews summarize these events well (Bulynko and O'Malley 2011; Burris et al. 2013; Kremoser et al. 2007).

A typical assay cascade starts with a biochemical, i.e., cell-free assay, where a recombinant full-length version or more typical the LBD in this case of FXR provides the backbone. From our own experience, the FXR-LBD can be expressed in normal *E. coli* expression systems without any special needs for posttranslational modifications or proper folding. The conformational switch, i.e., the changes adopted by Helix 11 and 12 upon agonist ligand binding, is exploited for typical biochemical assay formats. The most common one for nuclear receptors such as FXR is the homogeneous time-resolved fluorescence energy transfer (HTR-FRET) assay (Glickman et al. 2002). In the FRET format, a recombinantly expressed

FXR-LBD is combined with a peptide of approx. 20 amino acids that contains an LXXLL sequence (where X can be any amino acid) taken from one of the known canonical nuclear receptor coactivators (e.g., SRC-1, TIF-2, NCoA3). The LXXLL sequence is able to interact with the agonist conformation where Helix 11 and 12 are coordinated by the agonist ligand. In order to elicit a FRET signal, an appropriate fluorescence donor and acceptor pair is needed. Typically, a europium cryptate, a rare earth metal aromatic system chelate, is used as a donor and a red light-emitting acceptor such as XL665, a phycobilliprotein, or allophycocyanin (APC) as an acceptor. N-terminally fused to the FXR-LBD is a generic tag, typically a Glutathione *S*-transferase (GST) domain, which is recognized by an anti-GST antibody conjugated to the Eu-cryptate. The coactivator peptide with the LXXLL-motif is typically biotinylated which allows for the use of a generic streptavidin-APC complex to bind to it and thereby provides a fluorescent tag. Once an agonist ligand induces the appropriate conformation, the LXXLL-coactivator peptide binds to the activated FXR-LBD bringing the peptide-biotin-streptavidin-APC complex into close proximity with the GST-anti-GST Eu-cryptate, thus generating a rather long-lived (i.e., a few milliseconds) FRET signal. This can be easily read out by appropriate fluorescent readers. The respective reagents are all commercially available. This FRET assay format is probably best-suited for the cost-effective and robust screening of compound libraries for novel FXR agonists.

Alternative biochemical formats are AlphaScreen® or scintillation-proximity assays (SPA). Whereas the AlphaScreen® setup is basically similar to the FRET setup with just using the generation of a short-lived singlet oxygen to induce light emission in a receptive bead in close proximity, the SPA is a classical ligand-binding assay. SPA just relies on the displacement of a radioactively (i.e., ^{3}H) labelled reference ligand. The radioligand induces scintillation signals when the FXR-LBD is bound to an appropriate scintillator bead or plate. Upon its displacement by a new incoming and unlabeled FXR agonist, the scintillation signal is weakened. Using appropriate dose-response curves and Scatchard analysis, one can derive a binding constant K_s for the new ligand. However, the key disadvantage of the SPA format is that it does not discriminate between agonists, partial agonists, and antagonists which can also turn into an advantage, of course, if such type of ligands is sought.

The biochemical assays are normally used as a primary screening tool, while cellular reporter assays are used as secondary assays for hit verification and also to determine generic properties of identified hits such as cell membrane permeation or cytotoxicity. These cellular reporter assays employ the transcription factor properties of nuclear receptors. FXR recognizes a certain DNA hexamer motif, a so-called IR-1 element, which is an inverted repetition of the ACCTCA with one nucleotide as a spacer in between. Therefore, it is possible to take a part of an active promoter region from a known FXR direct target gene, e.g., IBABP (ileal bile acid-binding protein) or SHP (small heterodimer partner), and put it in front of a reporter gene that will then be transcribed in an FXR-dependent manner. In an ideal setup for such a direct reporter gene (DR) assay, one has a plasmid encoding full-length FXR and RXR and another one with the appropriate FXRE (IR-1 containing) promoter-reporter construct (typically *Firefly* luciferase). Bringing both into a cell line (e.g., HEK293

cells) by transient transfection yields a fully functional cellular FXR assay. An even more simplified yet fully functional reporter assay, the so-called Gal4-hybrid or mammalian-one-hybrid format, uses only the FXR-LBD fused to the DNA-binding part of the Gal4 transcription factor from yeast. The resulting hybrid protein is inactive, but upon FXR ligand binding will recruit the necessary coactivators to initiate transcription from Gal4-promoter containing genes. The same luciferase reporter constructs can be used in this format just with a Gal4 promoter instead of an FXRE-containing one.

As a tertiary assay which would be even closer to the native state of FXR, one can use cell lines that endogenously express FXR in an appropriate context and where activation of FXR by an agonist would drive transcription of native direct target genes. Unfortunately, most liver-derived cell lines such as HepG2 or HuH7 human hepatoma cells have only little functional FXR under normal conditions, but they can be "boosted" by just adding full-length recombinant FXR by transfection and selection for stably overexpressing clones.

With regard to cellular assays, it should be noted that cell membrane permeability is a key requirement for appropriate readouts for potency and efficacy of FXR agonists. That can be simply monitored by testing the natural ligands such as chenodeoxycholic acid (CDCA) and its tauro conjugate tauro-CDCA. Even in the first papers that describe FXR as a bile acid sensor (Makishima et al. 1999; Parks et al. 1999), it is noted that for tauro-CDCA to elicit a signal in a cellular reporter assay, a functional bile acid transporter has to be added. The Na^+-taurocholate cotransporting polypeptide (NTCP, SLC10A1) is the natural bile acid transporter for the import of conjugated bile acids from the sinusoids into hepatocytes and is therefore well suited to provide this functionality when co-transfected into the reporter assay cell line.

Is a bile acid transporter needed also for synthetic FXR agonists, as they are all structurally very unrelated to the bile acid steroid nucleus?

This question can only be answered when the pharmacokinetics and the in vivo metabolic fate of synthetic FXR agonists are carefully analyzed. We have performed systematic analysis of plasma pharmacokinetics and liver-to-plasma compound level ratios comparing homologous series of isoxazole "hammerhead" type of compounds in mice (Kinzel et al. 2016) and collected bile from the gallbladder of FXR agonist-treated mice to compare the metabolite spectrum of FXR agonists in plasma, liver, and bile. It should be noted that all "hammerhead"-type FXR agonists contain an acidic function and the terminal end of the stretched molecule, typically a normal, mostly aromatic carboxylic acid, but sometimes (i.e., Enanta compound from Fig. 1) this is replaced by an acid isostere such as a tetrazole or an acyl-sulfonamide. We have found that isoxazole-type FXR agonists with carboxylic acids are often conjugated with taurine in vivo which is one special form of Phase II metabolization. The degree of taurine conjugation varies with the degree of lipophilicity of such compounds; the more lipophilic, the more taurine-conjugated. In this aspect, this type of synthetic FXR agonist seems to mimic the conformation of hydrophobic, unconjugated bile acids since they are recognized by the two bile acid-conjugating

enzymes Bile acyl-CoA synthetase (BACS) and Bile acyl-CoA-aminotransferase (BAAT) to about the same extent as natural unconjugated bile acids.

As mentioned before, taurine conjugates of bile acids but also of synthetic FXR agonists cannot penetrate the cell membrane due to the permanent negative charge of the taurine sulfonic acid function. However, when adding NTCP to a cellular reporter assay, taurine-conjugated compounds show about the same potency and efficacy compared to their unconjugated counterparts. The reason for this is that the taurine is added to the carboxylic acid which is located just to the opposite of the ligand molecule where the key agonist interaction between Helix 11 and 12 takes place. It is the threefold-substituted isoxazole "hammerhead" that locks the active conformation, whereas the acid part is engaged in some hydrogen bond interaction and potentially ionic interactions with arginine residues at the other side of the ligand-binding pocket [see Gege et al. (2014) and references therein for an in-depth discussion]. An extension of the acid by an amide-type prolongation is well permitted and the taurine therefore well accepted. The sulfonic acid part reaches out into the water environment and does not need to be desolvated which means there is no free enthalpy cost for the ligand binding of a taurine conjugate as opposed to an unconjugated carboxylic acid.

However, unlike taurine- or glycine-conjugated bile acids which have a very high degree (>95%) of reuptake in the intestine and thus enterohepatic circulation, we have found that taurine-conjugated synthetic "hammerhead"-type of isoxazoles are not bioavailable. Thus it seems as if they are not accepted as substrates of the intestinal bile acid uptake transporter apical sodium-dependent bile salt transporter (ASBT/IBAT).

In summary, synthetic FXR agonists, despite their chemical unrelatedness to the bile acid steroid nucleus, may adopt features of bile acid-type metabolization, i.e., Phase II metabolization such as taurine conjugation, acyl glucuronidation, and less Phase I metabolization, e.g., hydroxylation. However, the degree of "chemical mimicry" varies in that they seem to be accepted as substrates for hepatocyte-borne bile acid transporters, e.g., the sinusoidal NTCP or the canalicular BSEP but not necessarily for intestinal transporters. There are also synthetic FXR agonists that do not show a bile acid-like PK behavior. It seems as if the individual differences depend on the exact conformation of the ligand in the pocket but also on the degree of lipophilicity of the ligand (the more hydrophobic, the more similar to the bile acid pattern).

Another important aspect from the PK and tissue distribution point of view is that the main sites of action of FXR agonists by definition are the small intestine and the liver. Carboxylic acid-bearing synthetic FXR agonists are the ones that are most active in vivo, but they are also prone to be actively taken up by liver transporter such as NTCP as discussed before or more unspecific organic anion transporting polypeptide (OATP) transporters. We have observed huge variations in the plasma-to-liver ratios of different synthetic FXR agonists (Kinzel et al. 2016), but a prevalent motif is that FXR agonists that tend to be enriched in the liver turn out to show very potent in vivo effects but this includes also the side effects (see discussion at the very end: intestinal versus liver activity of FXR agonists). This is

mentioned here because it means in consequence that plasma pharmacokinetics are misleading as guidance of active FXR agonist exposure and the liver residence time may be far longer than the actual plasma lifetime.

4 Applications for Nonsteroidal FXR Agonists: Preclinical Studies

Based on the biological roles of FXR in bile acid (BA) metabolism and whole-body energy homeostasis, FXR agonists are gaining attention as potential therapeutic agents in hepatobiliary disease. The following paragraph will highlight several preclinical studies and experimental models, focusing in particular on those relevant for currently ongoing clinical studies and indications.

4.1 Cholestatic Liver Diseases and BA Dysregulation

FXR is highly expressed in tissues that participate in BA metabolism such as the liver and intestine. Upon activation by conjugated and unconjugated bile salts, FXR regulates BA homeostasis by controlling genes involved in BA synthesis, secretion, conjugation, transportation, absorption, and detoxification (Mazuy et al. 2015; Wang et al. 2008a). Thereby FXR critically regulates bile formation and BA enterohepatic circulation. BAs and FXR play a pivotal role in regulating hepatic inflammation and regeneration as well as in regulating extent of inflammatory responses, barrier function, and prevention of bacterial translocation in the intestine. Thus, FXR has been considered as a promising target for the treatment of disorders of the biliary and gastrointestinal tract such as cholestatic liver diseases and inflammatory bowel disease (IBD) (Gadaleta et al. 2010).

The chronic cholestatic diseases PBC and PSC are characterized by defective BA flow from the liver to the intestine. The impaired bile secretion and transport result in intrahepatic accumulation of bile acids and cause fibrosis, inflammation, and cirrhosis. Additionally, the amount of BAs within the intestinal lumen is often decreased, which may lead to insufficient FXR-dependent FGF19 secretion by the enterocyte, thereby inducing a vicious circle of increased hepatic BA synthesis and progressive liver damage. FXR activation reduces the BA pool size, which represents one of the most important factors in cholestasis, by downregulating cytochrome P450 family 7 subfamily A member 1 (CYP7A1) expression via a synergistic mechanism that involves hepatic and intestinal FXR (Inagaki et al. 2005; Kim et al. 2007; Sinal et al. 2000). Several animal models support the role of FXR in the pathogenesis of cholestasis. In two models of acute cholestatic liver injury in rats (bile duct ligation (BDL) and α-naphthylisothiocyanate (ANIT) toxicity), the synthetic FXR agonist GW4064 markedly reduces bile duct proliferation, inflammation, and liver injury (Liu et al. 2003). Moreover, both GW4064 and OCA were able to protect rats from ethinyl-estradiol-induced cholestasis (Fiorucci et al. 2005a). Back then, the proposed protective effect of FXR was ascribed only to the reduced expression of CYP7A1

and NTCP and the induction of multidrug resistance-associated protein 2 (MRP2) and bile salt export pump (BSEP) in the liver. Today, evidence has shown a critical involvement of intestinal FXR activation leading to an additional repression of liver CYP7A1 expression, thereby further reducing BA pool size. Furthermore, FXR has been implicated to protect against pathogen-associated molecular patterns (PAMP) recognition and inflammatory signaling via downregulation of NF-kB pathways (Chignard and Poupon 2009; Wang et al. 2008b) adding an anti-inflammatory component to the pro-choleretic effects of FXR activation.

4.2 Inflammatory Bowel Disease (IBD)

IBD represents a group of disorders characterized by chronic intestinal inflammation. Currently, it is believed to result from dysregulation of the mucosal immune system, compromised intestinal epithelial barrier function, and an atypical, unhealthy gut microbiome (Podolsky 2002). Implicating FXR function as an important determinant in IBD, chemical-induced intestinal inflammation was shown to be reinforced in $Fxr^{-/-}$ mice, and genetic variation of FXR is reported to be associated with human IBD (Attinkara et al. 2012). Moreover, the steroidal FXR agonist OCA was able to protect mice from chemical-induced intestinal inflammation (Gadaleta et al. 2011). FXR alleviates inflammation and preserves the integrity of the intestinal epithelial barrier. Activated FXR also limits bacterial overgrowth and prevents bacterial translocation in the intestinal tract (reviewed in Ding et al. 2015; Gadaleta et al. 2010).

4.3 Metabolic Liver Diseases Nonalcoholic Fatty Liver Disease (NAFLD)/NASH

NAFLD presents a spectrum of liver diseases initiated with excess accumulation of lipids in the hepatocytes. NAFLD starts with simple benign hepatic steatosis, progresses further to NASH, characterized by liver steatosis in addition to signs of inflammation and ballooning of the liver cells, and ultimately leads to NASH-induced liver fibrosis and cirrhosis, the common end stage of most chronic liver diseases. At the stage of NASH, the risk of hepatocellular carcinoma (HCC) is substantially increased (Torres et al. 2012). From the etiology, NASH is closely linked to features of the metabolic syndrome such as obesity, hypertriglyceridemia, low high-density lipoprotein levels, hypertension, and elevated fasting plasma glucose (Yki-Järvinen 2014), and it is suggested that a bi-directional mutual relationship between NASH and various components of metabolic syndrome or even overt T2D exists (Lonardo et al. 2018). Along with the tight control of BA metabolism exerted by FXR, accumulating data demonstrate that FXR also plays an essential role in maintaining lipid and glucose homeostasis (Cariou et al. 2006; Lee et al. 2006; Ma et al. 2006; Stayrook et al. 2005; Zhang et al. 2006) (reviewed in Lefebvre et al. 2009; Zhang and Edwards 2008) and that modulation of FXR can

have significant influence on metabolic homeostasis. FXR is regarded as a promising therapeutic target for obesity and NASH, and the activation of FXR showed beneficial effects on various metabolic diseases, including fatty liver diseases, T2D, dyslipidemia, and obesity (Chávez-Talavera et al. 2017; Teodoro et al. 2011).

Fxr$^{-/-}$ mice display elevated serum cholesterol and triglyceride levels and an excessive accumulation of fat in the liver (Lambert et al. 2003; Sinal et al. 2000). Furthermore, they develop signs of insulin resistance as shown by hyperglycemia, impaired glucose tolerance, and severely blunted insulin signaling in both liver and muscle (Stayrook et al. 2005; Zhang et al. 2006). Activation of FXR by BAs or synthetic agonists lowers plasma triglyceride levels by a mechanism that involves the repression of hepatic transcription factor sterol regulatory element-binding protein 1c (SREBP-1c) expression and its lipogenic target genes in mouse primary hepatocytes and liver (Watanabe et al. 2004; Zhang et al. 2009). In the Zucker (*fa/fa*) rat, a model for NAFLD, where liver steatosis, diabetes, insulin resistance, and obesity occur due to a loss-of-function mutation of the leptin receptor, FXR activation by OCA protected against liver steatosis, body weight gain, and reversed insulin resistance (Cipriani et al. 2010). In addition, WAY-450 attenuated fructose-induced hepatic steatosis through the suppression of inflammation and the hepatic lipid droplet protein in mice (Liu et al. 2014). GW4064 improved insulin resistance in *ob/ob* mice, a genetic obesity model, and differentiated 3T3-L1 adipocytes displayed an enhanced insulin signaling and insulin-stimulated glucose uptake upon FXR agonist treatment (Cariou et al. 2006). Fexaramine also reduced obesity and promoted adipose tissue browning in mice (Fang et al. 2015). Beyond the individual metabolic effects, FXR agonists also improved liver histology in all parameters with reductions in fibrosis and steatosis and anti-inflammatory effects, in addition. In mice on methionine and choline deficient diet, WAY-450 reduced liver inflammation and fibrosis without triglyceride accumulation (Zhang et al. 2009). Furthermore, in STAM™ mice, a chemical-induced NASH model that develops manifest NASH at 8 weeks, Px-102 treatment was shown to improve liver histology, along with reduced inflammation and lipid disposition (Kremoser et al. 2011). The successor molecule to Px-102, cilofexor, also led to a significant decrease in liver steatosis and fibrosis in C57BL/6 mice kept on a fast-food diet (Liles et al. 2016).

4.4 Liver Fibrosis, Cirrhosis, and Portal Hypertension

In NAFLD, the progression from simple steatosis to NASH is determined by the initiation of the fibrotic response. As in NAFLD patients, it was shown that fibrosis stage but not NASH predicts clinical outcomes in terms of mortality (Dulai et al. 2017; Hagström et al. 2017). This is why fibrosis reduction or NASH resolution without worsening of fibrosis is the clinical endpoint which is accepted by the regulatory agencies FDA and EMA (Filozof et al. 2017; Hannah et al. 2016). Hepatic stellate cells (HSCs) are the main regulators of extracellular matrix (ECM) production and play an essential role in the development of fibrosis, and activation of HSCs

is critical for initiation and progression of liver fibrosis (Tsuchida and Friedman 2017).

FXR expression was reported to be low in human HSCs (Fickert et al. 2009), but the roles of FXR in HSC biology were demonstrated as treatment with OCA increased the PPARγ mRNA levels in HSCs and in rodent models of liver fibrosis, leading to the inhibition of the HSCs activation (Fiorucci et al. 2004, 2005c). In addition, activation of the FXR-SHP regulatory cascade by OCA mediated inhibition of HSCs and promoted the resolution of liver fibrosis (Fiorucci et al. 2005b). Furthermore, in fibrotic liver tissues of humans and mice, FXR expression was reduced, and activation of FXR reduced mitochondrial dysfunction and oxidative stress and increased hepatocyte survival by repression of miR-199a-3p targeting liver kinase B1 (LKB1) (Lee et al. 2012). A recent study reported that inducing FXR signaling by GW4064 activated the hepatic inositol-requiring enzyme 1α/X-box binding protein 1 pathway of the unfolded protein response (Liu et al. 2018), suggesting the possible role of FXR as regulator for endoplasmic reticulum (ER) stress, a stimulator of fibrosis development. FXR activation by GW4064 in isolated rat HSCs led to an inhibition of the endothelin-1-mediated contraction and trans-differentiation (Li et al. 2010) and increased the miR-29a promoter activity responsible for the inhibition of ECM production in HSCs (Li et al. 2011).

There is a gradual transition from a fibrotic NASH liver toward a cirrhotic liver. A key hallmark of cirrhosis is portal hypertension, i.e., the increase in the hepatic vein pressure gradient (HVPG) as a reflection of reduced blood flow through the sinusoids of the liver. The HVPG is a good predictor of mortality, and thus reduction of HVPG to levels $<$10–12 mmHg is clinically desired (Ripoll et al. 2007) (reviewed in Bosch and Iwakiri 2018). FXR agonists, both OCA and the nonsteroidal Px-102, have demonstrated to reduce the HVPG in rat models of cirrhosis and portal hypertension through various mechanisms including endothelial nitric oxide synthase (eNOS)-mediated sinusoidal vasodilation and reduction of the vasoconstrictive factors endothelin-1 and p-Moesin (Schwabl et al. 2017; Verbeke et al. 2014).

4.5 Cancer

Hepatocellular carcinoma (HCC) often occurs on the basis of chronic liver inflammation or fibrosis. There is a growing body of evidence indicating that FXR is involved in carcinogenesis. *Fxr*$^{-/-}$ mice were found to spontaneously develop liver tumors as they age (Yang et al. 2007), while selective activation of intestinal FXR protected mice against development of HCC (Modica et al. 2008). In a diethylnitrosoamine (DEN)-mediated liver cancer mouse model, GW4064 was found to reduce the expression of oncoprotein gankyrinin preventing liver cancer development (Jiang et al. 2013). Furthermore, stable overexpression on FXR or activation by Px-102 led to transcriptional induction of N-myc downstream-regulated gene 2 (NDRG2) a tumor suppressor and reduced liver tumor growth in an orthotopic xenograft model in nude mice (Deuschle et al. 2012). In *Abcb4*$^{-/-}$

mice, which are characterized by hepatic accumulation of BA and development of HCC, steroidal dual FXR/TGR5 agonist INT-767 administration significantly reduced the number and size of HCC nodules (Cariello et al. 2017). As FXR maintains BA pool size and composition within a physiological range and elevated BAs are considered as tumor-promoting factors in colorectal cancer development (Bernstein et al. 2005; Debruyne et al. 2001), an involvement for FXR was also implicated in the modulation of intestinal tumorigenesis. FXR mRNA expression was decreased in different colorectal adenoma and carcinoma cell lines (De Gottardi et al. 2004), and FXR deficiency led to significantly increased sizes and numbers of tumors in murine intestine tumorigenesis models APC^{min} mice and azoxymethane (AOM)-induced colon cancer (Maran et al. 2009). Moreover, activation of FXR induced a proapoptotic program in the differentiated normal colonic epithelium as well as transformed colonocytes and is thereby hypothesized to remove genetically altered cells, which may otherwise progress to complete neoplastic transformation (Modica et al. 2008). In human colon carcinomas tissues and human cell lines, FXR expression was markedly reduced and might be associated with an adverse prognosis (Lax et al. 2012). Furthermore, it was shown that early in the development of human colon carcinoma, FXR is silenced by DNA methylation and Kirsten rat sarcoma 2 viral oncogene homolog (KRAS) signaling (Bailey et al. 2014). Together, these clues suggest that FXR plays a key role in the pathogenesis of different carcinomas and that restoration of FXR expression or FXR activation could prevent tumor development or progression. In contrast to this tumor-preventive role of FXR, several studies implicate the FGF19-FGFR4 signaling axis as a tumor promoter or at least as a marker of HCC (Zhou et al. 2014) and colon carcinoma. The expression of intestinal FGF19 is controlled by FXR. $Fgf15^{-/-}$mice showed less tumors and histological neoplastic lesions compared to wild-type mice, and the hepatocellular proliferation was reduced in $Fgf15^{-/-}$ mice, which also expressed lower levels of the HCC marker alpha-fetoprotein (AFP) (Uriarte et al. 2015). In transgenic mice overexpressing human FGF19, increased hepatocellular proliferation was observed, and HCC development was evident within 12 months (Nicholes et al. 2002), while clinically it has been shown that FGF19 overexpression correlates with HCC progression and poorer progression (Miura et al. 2012). However, other studies have shown that it is not FGF19 but the overexpression of FGFR4 that is associated with poor clinical prognosis in HCC (French et al. 2012; Ho et al. 2009), prostate cancer (Murphy et al. 2010; Wang et al. 2004), cholangiocarcinoma (Xu et al. 2014), and breast cancer (reviewed in Lang and Teng 2019). Understanding the balance between pro- and anti-tumorigenic properties of FXR activation and unifying these findings in a consistent model how FXR impacts carcinogenesis require more preclinical and clinical validation. And upon refining our understanding, it will become clearer if we need FXR agonists or antagonists for cancer applications.

5 Applications for Nonsteroidal FXR Agonists: Clinical Effects

In the past decade, NASH has emerged as a leading cause for chronic liver disease. NAFLD and NASH represent a complex spectrum of liver diseases, but its stage and severity can be characterized by histopathological determination of the degrees of (1) steatosis, (2) cytoskeletal damage (hepatocellular ballooning), (3) lobular inflammation, and (4) fibrosis (Bedossa 2017). As NAFLD is strongly associated with obesity and diabetes, it is considered to be the hepatic manifestation of the metabolic syndrome and of T2D (Hu et al. 2017; Loomba and Sanyal 2013). The majority of subjects with NAFLD are asymptomatic and are diagnosed incidentally. While patients with simple steatosis suffer no negative consequences in terms of life expectancy, the fraction that progress to NASH face a worse outcome (Torres et al. 2012). Despite growing prevalence, the factors influencing NAFLD development and subsequent progression to NASH, liver fibrosis, cirrhosis, and HCC are still not fully elucidated. For late-stage NASH patients, often the only therapy option left is liver transplantation leading to the assessment of NAFLD soon becoming the leading cause of liver transplantation worldwide (Byrne and Targher 2015).

As of today, there are no approved drugs for the treatment for NAFLD and NASH. But as a large number of emerging therapies are being evaluated in the clinic (extensively reviewed in Konerman et al. 2018), FXR agonists are among other nuclear receptor modulators such as thyroid hormone receptor beta or PPAR agonists, the most promising therapeutic agents for NASH (Schaap et al. 2014). Among other metabolic changes, NASH patients also have an altered BA profile. The strong impact on BA metabolism exerted by FXR agonists is also expected to have beneficial effects (Puri et al. 2018).

The most advanced FXR agonist in clinical development is OCA, a derivative of the natural ligand CDCA. The clinical trials with OCA have validated FXR as target and demonstrated the clinical potential of FXR agonists to treat hepatic steatosis, inflammation, and fibrosis while increasing insulin sensitivity (Mudaliar et al. 2013; Neuschwander-Tetri et al. 2015). Currently, two Phase III trials are ongoing evaluating the effects of OCA in non-cirrhotic NASH subjects (NCT02548351) and in adults with compensated cirrhosis due to NASH (NCT03439254). As OCA is also under clinical investigation for PBC and while it was consequently more broadly dosed in patients, it was found that hepatically impaired patients, in particular, bear the risk of developing severe side effects. This was attributed to a non-adjustment of the dose to liver function and could occur even when treated with very low doses of OCA once a week.[1,2] Following in OCAs footsteps, a number of nonsteroidal FXR agonists are in clinical development, promising a better safety

[1]FDA Drug Safety Communication. https://www.fda.gov/Drugs/DrugSafety/ucm594941.htm.

[2]FDA Adverse Event Reporting System (FAERS) Public Dashboard. https://fis.fda.gov/sense/app/777e9f4d-0cf8-448e8068-f564c31baa25/sheet/45beeb74-30ab-46be-8267-5756582633b4/state/analysis.

Table 1 Overview of clinical trials in NASH for nonsteroidal FXR agonists

Drug	Indication	Company	Highest developmental stage/clinical trial identifier
Obeticholic acid (INT-747)	NASH	Intercept Pharmaceuticals	Phase III/NCT02548351
Obeticholic acid (INT-747)	NASH, compensated cirrhosis	Intercept Pharmaceuticals	Phase III/NCT03439254
Px-104	NAFLD	Phenex Pharmaceuticals	Phase IIa (discontinued)/NCT01999101
Tropifexor (LJN452)	NASH	Novartis	Phase IIa/NCT02855164
Tropifexor (LJN452)	NASH	Novartis	Phase II combination trial with cenicriviroc/ NCT03517540
Nidufexor (LMB763)	NASH	Novartis	Phase IIa/NCT02913105
Cilofexor (GS-9674)	NASH	Gilead Sciences	Phase II/NCT02854605
EYP001	NASH	Enyo Pharma	Phase II/NCT03812029
MET409	NASH, IBD	Metacrine	Phase I; Homepage: *"We are currently conducting a Phase 1 clinical trial of MET409 in healthy volunteers"*

profile and more controllable PK compared to the bile acid-like OCA. An overview of the clinical evaluation of different FXR agonists in NASH is shown in Table 1.

Most clinical trials evaluate liver histopathology, more specifically the NAFLD activity score (NAS) which covers the three qualities steatosis, inflammation and hepatocyte ballooning, and the degree of liver fibrosis, typically assessed by the METAVIR scale, as main outcomes.[3] Furthermore, current data suggest that different approaches may be beneficial in subgroups of patients with NASH. Since the phenotype of NASH develops in the context of different genetic predispositions and environmental exposures, it is most likely that no single therapy will reverse NASH in all patients. For this reason, companies are evaluating combination therapies pairing nonsteroidal FXR agonists with other compounds targeting different pathways in the NASH pathophysiology.

The FLINT trial represents the first finished Phase II trial for NASH. This multicenter, randomized trial evaluated 72 weeks of OCA treatment (25 mg) versus placebo in patients with non-cirrhotic NASH (Neuschwander-Tetri et al. 2015). Here a total dose of 25 mg OCA daily for 72 weeks resulted in improvements in the composite NAS and fibrosis, while severe pruritus reported in 33 of 141 (23%) OCA-treated patients ($p < 0.0001$ vs placebo), and an increase in total cholesterol

[3]FDA Noncirrhotic Nonalcoholic Steatohepatitis With Liver Fibrosis: Developing Drugs for Treatment-Guidance for Industry. https://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM627376.pdf.

and LDL cholesterol and a modest decrease in HDL cholesterol (changes peaked at 12 weeks of treatment and then decreased slowly and stabilized) were discerned as possible therapy-limiting side effects.

At the beginning of 2019, pharma companies Gilead Sciences, Novartis, and Enyo Pharma were conducting Phase II trials in NASH patients testing nonsteroidal FXR agonists cilofexor, nidufexor, tropifexor, and EYP001.

First data for cilofexor were reported from a Phase II study with 140 NASH patients, treated with cilofexor at 100 or 30 mg or placebo orally, once daily for 24 weeks (Patel et al. 2018). At 24 weeks, a significant decrease in hepatic steatosis of at least 30% assessed by magnetic resonance imaging-proton density fat fraction (MRI-PDFF) was observed in 38.9% of patients treated with cilofexor 100 mg ($p = 0.011$ vs placebo), 14% treated with cilofexor 30 mg ($p = 0.87$), and 12.5% treated with placebo. Further significant observations in the cilofexor-treated patients were improvements in liver biochemistry (serum gamma-glutamyltransferase (GGT)) and response pharmacodynamic markers (serum 7α-hydroxy-4-cholesten-3-one (C4) and BAs). Cilofexor was generally well tolerated, and changes in lipid profile and glycemic parameters did not differ between cilofexor and placebo-treated patients. However, moderate to severe pruritus occurred in 14% of patients in the cilofexor 100 mg arm compared to 4% in the cilofexor 30 mg and placebo arms.

A separate Phase II study (ATLAS) is investigating treatment with cilofexor alone or in combination with investigational NASH drugs acetyl-CoA carboxylase (ACC) inhibitor firsocostat (GS-0976) and apoptosis signal-regulating kinase 1 (ASK-1) inhibitor selonsertib, in patients with advanced fibrosis due to NASH. This randomized, double-blind 52-week trial will evaluate improvement in fibrosis without worsening of NASH, adverse events, and plasma laboratory abnormalities. First data from a proof-of-concept study using cilofexor were presented at the International Liver Congress 2018 (Lawitz et al. 2018). The study also included combination treatment with selonsertib and firsocostat. A 12-week treatment regime was evaluated for safety and efficacy in patients with NASH and F2-F3 fibrosis. MRI-PDFF evaluation showed a median relative change to baseline −15.6% for cilofexor and beneficial effects on liver biochemistry (ALT –29.7%, GGT –19.3%). Fibrosis was not evaluated.

Further interim results were reported on a Phase II study assessing several doses of tropifexor for safety, tolerability, and efficacy in NASH patients (FLIGHT-FXR) after 12 weeks of therapy (Sanyal et al. 2018). At 12 weeks, a significant decrease in hepatic steatosis of at least 5% assessed by MRI-PDFF was observed in 33.3% of patients treated with 90 μg, 27.8% treated with 60 μg, and 14.6% treated with placebo. Furthermore, a dose-response decrease in GGT levels was observed as well as increases in FGF19. Adverse events and pruritus were reported to be comparable between 90 μg tropifexor arm and placebo. However a mild dose-related increase in LDL_c and decrease in HDL_c were observed in the 60 and 90 μg arms. As reported for the cholesterol changes during the FLINT trial (Neuschwander-Tetri et al. 2015), these effects were more prominent after initiation of treatment (2 weeks) and then slowly declined.

Tropifexor is also under investigation in a randomized, double-blind combination trial (TANDEM) with cenicriviroc, an investigational C-C chemokine receptor-type (CCR) 2/5 inhibitor (Stringer 2019). This combination therapy headed by Novartis should address metabolic, anti-inflammatory, and anti-fibrotic pathways involved in NASH. The 48-week trial will assess improvement in liver histology and occurrence of adverse events and serious adverse events in single and combination arms in approximately 200 patients with NASH and liver fibrosis.

Figure 9 compares data from Phase II trials on hepatic steatosis reduction. Due to profound differences in the individual trial designs, a full evaluation whether nonsteroidal FXR agonists are indeed superior to bile acid-like structures like OCA is not possible. The same caveat is true for the comparison of possibly dose-limiting side effects shown in Table 2 as no direct comparative clinical data are available.

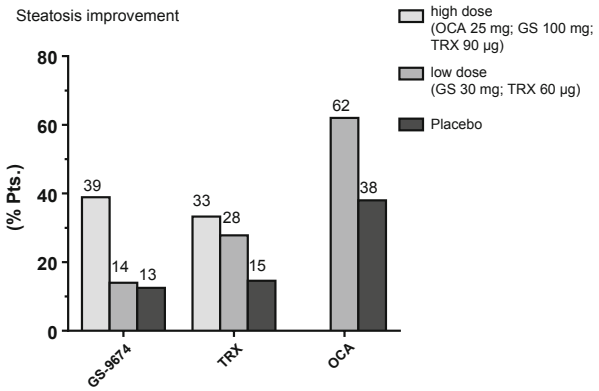


Fig. 9 Improvement of hepatic steatosis with FXR agonists. Results are from separate studies and do not represent a reflection of head-to-head direct comparison of listed agents on outcomes of interest. Trials for individual treatment agents employed different enrollment criteria and durations of therapy, and the primary endpoint definitions were not identical (TRX, tropifexor; GS, cilofexor)

Table 2 Comparison of possible dose-limiting side effects with FXR agonists

	Patients with moderate to severe pruritus (%)	HDL lowering to baseline	LDL increase to baseline
OCA 25 mg	23	Yes	Yes
Placebo	6		
Tropifexor 90 µg	8	Yes/mild	Yes
Tropifexor 60 µg	14	None	Yes
Placebo	7		
Cilofexor 100 mg	14	None	None
Cilofexor 30 mg	4	None	None
Placebo	4		

Results are from separate studies and are not meant as a head-to-head direct comparison of listed agents. Trials for individual treatment agents employed different enrollment criteria and durations of therapy, and the primary endpoint definitions were not identical. Data taken from Neuschwander-Tetri et al. (2015), Patel et al. (2018), and Sanyal et al. (2018)

However, the available evidence from clinical trials with nonsteroidal FXR agonists suggests that these class of substances may hold the potential to bring forth better FXR-targeting drugs with improved pharmacological actions and reduced adverse effects in particular in terms of cholesterol metabolism and pruritus. In conclusion, currently ongoing Phase II trials with nonsteroidal FXR agonist will have to prove if this class of substances is indeed superior in terms of observed side effects while retaining efficacy. This is of utmost importance, since NASH is (1) a largely asymptomatic disease and (2) future treatment will probably be long-term or even lifelong. Drugs with an onerous side effect profile are not acceptable, and potential treatment options shall not adversely impact cardiovascular risk, in particular, as this is the most common cause of death in patients with NASH.

As a consequence of the general hepatoprotective role of FXR as a master regulator of BAs, glucose, and lipid homeostasis, FXR also has been suggested as a promising pharmacological target in cholestatic liver diseases (recently reviewed in Goldstein and Levy 2018). PSC and PBC are the most common immune-mediated chronic cholestatic liver diseases leading to cirrhosis and liver failure. In particular for PSC there is an unmet need for effective medical treatments; as of today the only curative therapy is liver transplantation reserved for those with end-stage liver disease (Goldstein and Levy 2018). The first-line treatment for PBC is ursodeoxycholic acid (UDCA); it has choleretic and immunomodulatory properties and stimulates biliary bicarbonate secretion (Copaci et al. 2005). While UDCA is very well tolerated and can ultimately improve survival free of liver transplantation, the treatment offers an unfavorable response rate as approximately 40% of patients do not respond to UDCA and are at risk for progression (Corpechot et al. 2008).

The rationale for the use of FXR agonists in cholestatic liver diseases is that FXR activation is postulated to reduce toxic bile production and induce secretion through FXR-mediated pathways. Activation of FXR inhibits CYP7A1 (the rate limiting step of bile acid synthesis) directly, through translational activation of SHP, as well as indirectly, through the release of FGF19, which binds to FGFR4 on hepatocytes and leads to additional CYP7A1 inhibition (Inagaki et al. 2005; Rizzo et al. 2005). FXR upregulates (bile salt) transporters BSEP, MDR2/3, MRP2, and OSTα/β, further decreasing hepatocellular bile concentrations through increased canalicular secretion (Ananthanarayanan et al. 2001; Boyer et al. 2006; Kast et al. 2002). These effects should result in a limited accumulation of toxic BAs within the hepatocyte, thus reducing liver injury and potentially ameliorating inflammation of bile ducts.

OCA was granted conditional approval in May 2016 for treatment of PBC patients that were exhibiting an inadequate response to UDCA. It should be prescribed either in conjunction with UDCA or as single therapy if UDCA is not tolerated. This approval was based on results of the POISE trial, where 216 patients with PBC and an inadequate response to UDCA received either placebo, 5 mg, or 5 mg titrated to 10 mg OCA. After 12 months of treatment, 47% in the 5 mg OCA group and 46% in the 5–10 mg OCA group compared to 10% in the placebo group met the primary endpoint (serum alkaline phosphatase (ALP) $<1.67 \times$ ULN with a reduction of $\geq 15\%$ from baseline, normal total bilirubin) (Nevens et al. 2016). The most common side effect of OCA was a dose-dependent development of itching, and

use of OCA was also associated with a dose-dependent reduction in HDL_c. While OCA is promising in terms of its ability to significantly decrease ALP levels in PBC patients and possibly improve survival free of liver transplantation, a follow-up with studies is necessary to examine the long-term effects of OCA therapy and validate its suitability for patients with more advanced liver disease.

In regard to the use of OCA in PSC, the AESOP trial, a Phase II trial for dose-finding and evaluation of the efficacy and safety of OCA, looked at the effect of 24 weeks OCA treatment compared to placebo in 77 patients with PSC. The primary endpoint of the AESOP trial was the mean change in serum ALP levels. A statistically significant decrease in baseline ALP of 22% in both the low-dose (1.5–3 mg) and high-dose (5–10 mg) groups was observed (Kowdley et al. 2018; Larusso et al. 2018), and a long-term extension phase is ongoing. While encouraging, further trials are needed to assess if these results translate into a clinically significant endpoint such as increased time to transplantation or death.

Nevertheless, the efficacy demonstrated by OCA in the treatment of PBC has established FXR as a valuable therapeutic target, and different nonsteroidal FXR agonists are currently tested in PBC and PSC trials. Trials for PBC and PSC are summarized in Table 3.

Currently, three other FXR agonists are undergoing Phase II testing for PBC. These novel molecules include cilofexor and tropifexor and the steroidal FXR agonist EDP-305 by Enanta Pharmaceuticals (ClinicalTrials.gov identifiers: NCT03394924, not covered here).

Table 3 Overview of clinical trials in cholestatic and other liver diseases for nonsteroidal FXR agonists

Drug	Indication/ disease	Company	Highest developmental stage/clinical trial identifier
Obeticholic acid	PBC	Intercept Pharmaceuticals	Phase III approval (completed)/ NCT01473524
Obeticholic acid	PBC	Intercept Pharmaceuticals	Phase IV long-term outcome/ NCT02308111
Obeticholic acid	PSC	Intercept Pharmaceuticals	Phase II (completed)/NCT02177136
Obeticholic acid	Bile acid diarrhea (BAD)	Intercept Pharmaceuticals	Phase II/NCT01585025
Cilofexor (GS-9674)	PBC	Gilead Sciences	Phase II/NCT02943447
Cilofexor (GS-9674)	PSC	Gilead Sciences	Phase II/NCT02943460
Tropifexor (LJN452)	PBC	Novartis	Phase II/NCT02516605
Tropifexor (LJN452)	BAD	Novartis	Phase II /NCT02713243
EYP001	HBV	Enyo Pharma	Phase Ib (completed)/NCT03272009
MET409	Irritable bowel	Metacrine	Phase I; Metacrine homepage: *"We are currently conducting a Phase I clinical trial of MET409 in healthy volunteers"*
TERN-101	n.d.	Terns Pharmaceuticals	Phase I finished (see homepage)

For cilofexor, a Phase II, double-blind, placebo-controlled study evaluating the safety and tolerability of 30 mg and 100 mg cilofexor for 12 weeks in patients with PBC without cirrhosis is currently ongoing. Primary study outcomes include the incidence of treatment-emergent adverse events (AE) and serious adverse events (SAE) and laboratory abnormalities. Furthermore, a proof-of-concept Phase II trial assessing cilofexor in patients with PSC in an open-label fashion is also under investigation. This study enrolled 52 non-cirrhotic patients with PSC who received either 100 mg cilofexor, 30 mg cilofexor, or placebo orally once daily for 12 weeks. Dose-dependent reductions in liver biochemistry were observed, and after 12 weeks of treatment, 100 mg cilofexor led to significant improvements in liver biochemistry parameters, serum ALP (−21%; $p = 0.029$ vs placebo), GGT (−30%; $p < 0.001$), alanine aminotransferase (ALT) (−49%; $p = 0.009$), and aspartate aminotransferase (AST) (−42%; $p = 0.019$). In both groups treated with cilofexor, reduced serum levels of C4 were reduced compared with placebo (−23.2% in the 100 mg group, $p = 0.21$; and −30.5% in the 30 mg group, $p = 0.024$). Reductions in serum BAs were greatest with the 100 mg dose. Cilofexor was well tolerated, and the incidence of grade 2 or 3 pruritus was lower with cilofexor 100 mg (13.6%) and 30 mg (20%) compared with placebo (40%). There were no elevations in serum lipids (Trauner et al. 2019).

Tropifexor is also currently evaluated in a multipart, double-blind, placebo-controlled Phase II study to assess the safety, tolerability, and efficacy in patients with PBC. Part 1 includes a 28-week treatment and part 2 a 12-week treatment period with tropifexor. The primary study outcomes include safety and tolerability as well as changes in markers of cholestasis compared to baseline, while secondary objectives include evaluation of disease-specific quality of life and pharmacokinetics.

In a proof-of-concept study, treatment with OCA stimulated FGF19 release and decreased BA synthesis, producing clinical benefit in patients with bile acid diarrhea (BAD) after 2 weeks of treatment (stool frequency (24%; $p = 0.03$), stool form (14%; $p = 0.05$), and diarrhea index (34%; $p = 0.005$)) (Walters et al. 2015). Treatment was reported as well tolerated, and adverse effects included a change in lipids (increase in LDL_c), mild headache in 11% of patients, and no reports of pruritus. BAD is a common cause of chronic diarrhea, occurring as a primary condition or secondary to ileal disease or resection. Many patients have reduced levels of the ileal hormone FGF19, which acts as an inhibitory regulator of hepatic BA synthesis and secreted in response to FXR activation in the intestine. In primary BAD, impaired FGF19 production results in increased CYP7A1 expression and enhanced hepatic synthesis of BAs. In turn, this results in a larger BA pool with increased colonic delivery (Keely and Walters 2016). This proof-of-concept study supports a future role for FXR agonists in the treatment of BAD. Recently a double-blind, randomized, placebo-controlled crossover multiple-dose study of tropifexor to assess safety, tolerability, and efficacy in patients with primary BAD was concluded and is under evaluation. Still, for a more definitive assessment of long-term efficacy, further studies with larger numbers of patients are required both in patients with primary and secondary BAD.

Furthermore, as FXR controls the expression of NTCP, FXR agonism could be a viable principle to address viral hepatitis infections. NTCP has been demonstrated to be a functional receptor for HBV, mediating viral entry and consequent infection (Yan et al. 2012). By repressing NTCP, FXR agonism may block HBV entry and infection. Furthermore, FXR agonism could also directly inhibit HBV mRNA, DNA, and protein production and reduced covalently closed circular DNA pool size (Radreau et al. 2016). In cell culture, an enhanced effect was observed when combined with antiviral treatments entecavir or tenofovir (Joly et al. 2017). EYP001 is therefore explored as therapeutic option for HBV in a Phase Ib trial.

6 How to Differentiate Diverse FXR Agonists from Each Other?

In the previous section, we discussed the huge and diverse clinical potential of FXR agonists in various clinical indications, in general. The pioneering frontrunner FXR agonist in most applications except for HBV was OCA which represents a modified bile acid. There is a discussion in the literature that nonsteroidal FXR agonists might be superior to bile acid-type ones (Verbeke et al. 2017), and in a head-to-head comparison between Px-102 and OCA in a CCl_4-induced rat model of cirrhosis and portal hypertension, the nonsteroidal FXR agonists performed better indeed, but this could be a simple function of better in vivo potency (Schwabl et al. 2017). The key liabilities of OCA are the induction of pruritus in NASH patients, and in PBC patients, OCA even exacerbates the already existing disease-related pruritus. This is an adverse effect which directly affects the quality of patients' lives, while the changes in lipoprotein cholesterol toward a worse atherogenic index (defined as the ratio of HDL_c to LDL_c) pose more a long-term threat. A third issue of OCA is its uncontrolled PK since OCA as every bile acid is massively taurine- and glycine-conjugated in humans and in fact upon chronic dosing, these conjugated forms represent 90–95% of the plasma detectable metabolites of OCA. Genetic variations in the transporters involved in this enterohepatic cycling as well as food interactions and other individual variations may be responsible for the effect that the same dose of OCA, e.g., 10 mg daily, may result in substantially different plasma and FGF19 levels as a pharmacodynamics readout of FXR activation and this may give rise to over- and under-responders in the same dosing group (Marschall et al. 2012). Such effects can clearly be overcome by synthetic FXR agonists since it was shown that, e.g., Px-102 yielded a more drug-like PK behavior in Phase I healthy volunteers with acceptable low individual variations.

The basis of the lipoprotein cholesterol changes and of the pruritogenic effects is not well understood. Some publications related the pruritus to activation of TGR5, the other, GPCR-type bile acid receptor (Alemi et al. 2013; Lieu et al. 2014). Others make the increased levels of autotaxin, a phospholipase that releases lysophosphatidic acid (LPA) from circulating phospholipid substrates, responsible for bile acid mediated pruritus, in general (Kremer et al. 2010; Oude Elferink et al. 2011). Some research groups have described OCA as a submicromolar activator of TGR5

which might be one explanation (Fiorucci et al. 2014) of its pruritogenic potential but it cannot be the sole one since pruritus was also described as a mild side effect of cilofexor in the first Phase II study. Thus it is difficult to ascribe this adverse effect to a certain structural or molecular feature of a FXR agonist, and it needs to be demonstrated by future clinical study results how synthetic FXR agonists compare to steroidal ones in this regard.

Another point of differentiation very controversially discussed is the ratio of intestinal- versus liver-specific FXR activation. Fang et al. (2015) have shown that selective pharmacological activation of FXR by fexaramine, which has very limited bioavailability, is sufficient to elicit several of the aforementioned beneficial metabolic effects. Modica et al. point into the same direction by having shown that constitutive FXR expression just in intestines of mice is sufficient to yield potent anti-cholestatic effects (Modica et al. 2012). However, there are other publications that claim that antagonizing or inhibiting FXR in the intestine is what is needed to elicit desirable effects (Jiang et al. 2015; Kim et al. 2007) and that liver-selective FXR activation yields potent lipid reducing effects (Schmitt et al. 2015). The groups of Frank Gonzalez and John Chiang, the former responsible for promoting the idea that FXR antagonism is beneficial rather than agonism, very recently surprised by the finding that fexaramine indeed elicits adipose tissue browning and improves insulin sensitivity using a formerly not described pathway: intestinal FXR activation by fexaramine increases taurolithocholic acid and TGR5 as its receptor and that has an impact of the gut microbiome (Pathak et al. 2018). Pathak et al. ultimately make the modified gut microbiome responsible for the beneficial changes since the effects are gone when antibiotics are given to the mice.

The publication by Houten et al. (2007) sheds a light on the question of which tissue is physiologically more relevant in terms of FXR activation by endogenous ligands. Houten et al. have generated a transgenic mouse that harbors a luciferase under control of a FXR-responsive promoter. In this FXR-sensitive reporter mouse, only ileal FXR is active under normal physiological conditions. Liver FXR only becomes activated under cholestatic conditions of bile acid overflow, in this case simulated by bile duct ligation. Even GW4064 generates mainly an intestinal FXR signal although GW4064 is widely published to exert various beneficial metabolic effects.

Intestinal FXR induces FGF15 (in rodents) or FGF19 (in primates and humans) which circulates to the liver through the portal circulation where it can bind to FGFR4, a receptor tyrosine kinase. Activated FGFR4 induces signaling via the extracellular signal-regulated protein kinase (ERK) pathway to control glucose and glycogen synthesis. It also controls the activity of SHP to repress transcription of CYP7A1, the key pacemaker enzyme for the conversion of cholesterol into bile acids (Inagaki et al. 2005), probably by a c-Jun N-terminal kinase (JNK)-mediated pathway (Holt et al. 2003). Kong et al. have demonstrated that in mice, intestinal FXR activation via FGF signaling is sufficient to suppress transcription of both key enzymes, CYP7A1 for overall bile acid synthesis and CYP8B1 which introduces a third hydroxy group into the bile acid steroid nucleus, thus controlling the hydrophobicity of the bile acid pool (Kong et al. 2012). Liver FXR activation results

in upregulation of SHP which acts as a suppressor of CYP7A1 on the promoter of this gene (Goodwin et al. 2000; Lu et al. 2000); however, Kong et al. have found that liver FXR exerts stronger repressive control on CYP8B1 than on CYP7A1, whereas the intestinal FXR-FGF-FGFR4 axis has a stronger effect on CYP7A1 compared to CYP8B1. These data were mainly generated in mice, but if they could be extrapolated to humans, it had important functional meanings. De Boer et al. (2017) showed in an elegant study that FGF15 signaling plus liver FXR activation by Px-102 in mice changed the hydrophobicity of the bile acid pool and that this change led to a massive increase of transintestinal cholesterol excretion (TICE) which finally resulted in plasma cholesterol lowering affecting LDL as well as HDL cholesterol. The compound used in this study, Px-102, however, is also very active in the liver, and its effects on liver CYP8B1 suppression are likely responsible for changing the hydrophobicity of the bile acid pool. If one compares the changes in the bile acid pool composition between Px-102-treated mice (de Boer et al. 2017) with the pattern from fexaramine-treated mice (Fang et al. 2015), it becomes obvious that the intestinally restricted FXR agonist fexaramine induces by far less changes in the hydrophobicity of the bile acid pool also with only little increase in tauro-beta muricholic acid compared to Px-102. The fexaramine-treated mice show many beneficial metabolic effects but only little cholesterol lowering, whereas Px-102 led to a massive increase in fecal neutral sterol excretion and thus plasma cholesterol reductions (see also Hambruch et al. 2012).

In essence we tended to follow the hypothesis from Fang et al. that intestinal FXR activation is sufficient to elicit most of the desired metabolic effects but avoids that undesired changes in BA pool composition which are likely responsible for the HDL cholesterol-lowering effects. For Px-102, an FXR agonist found to be very active in the intestine as well as in the liver compared to GW4064 (Houten et al. 2007) and OCA, it was shown that liver FXR activation also leads to upregulation of SR-BI as the scavenger receptor for HDL_c and that this is one contributor to the apparent plasma HDL_c lowering on top of the cholesterol losses through intestinal excretion (Hambruch et al. 2012). Thus it was a reasonable assumption that more intestinally restricted FXR agonist could be void of the BA-induced side effects, potentially even of the pruritogenic effects if they were really BA related, but that such a compound would still retain most of the beneficial metabolic potential, in particular for the treatment of NASH.

Thus we have developed cilofexor which is in Phase IIb in clinical development in NASH, PBC, and PSC. Cilofexor, due to its specific tissue distribution and physicochemical properties, yields an intestinally biased FXR agonist which is still bioavailable but mostly lacks transcriptional activity in the liver. The detailed mechanisms how this can be achieved will be published soon (manuscript in preparation). By now it can be stated that cilofexor yields less HDL_c-lowering effects as determined by a 21-day Phase I study in healthy human volunteers (Myers et al. 2018) and that it yields decent antisteatotic effects with only little pruritogenic potential compared to OCA in a first Phase II clinical trial in NASH patients. If such an intestinally biased "designer" FXR agonist is really superior to other unbiased but very potent nonsteroidal FXR agonists such as tropifexor, it needs to

be proven in large-scale clinical studies which carefully evaluate the therapeutic index between the beneficial liver-protective effects and the adverse effects in terms of pruritus, undesired HDL_c to LDL_c changes, and the potential liver proliferation and cancerogenicity in animal studies.

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Potential of Intestine-Selective FXR Modulation for Treatment of Metabolic Disease

Tim van Zutphen, Anna Bertolini, Hilde D. de Vries, Vincent W. Bloks, Jan Freark de Boer, Johan W. Jonker, and Folkert Kuipers

Contents

T. van Zutphen · H. D. de Vries
Department of Pediatrics, University Medical Center Groningen, Faculty Campus Fryslân, University of Groningen, Groningen, The Netherlands

University of Groningen, Leeuwarden, The Netherlands

A. Bertolini · V. W. Bloks · J. W. Jonker
Department of Pediatrics, University Medical Center Groningen, Faculty Campus Fryslân, University of Groningen, Groningen, The Netherlands

J. F. de Boer · F. Kuipers (✉)
Department of Pediatrics, University Medical Center Groningen, Faculty Campus Fryslân, University of Groningen, Groningen, The Netherlands

Department of Laboratory Medicine, University of Groningen, University Medical Center Groningen, Groningen, The Netherlands
e-mail: f.kuipers@umcg.nl

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Abstract

Farnesoid X receptor controls bile acid metabolism, both in the liver and intestine. This potent nuclear receptor not only maintains homeostasis of its own ligands, i.e., bile acids, but also regulates glucose and lipid metabolism as well as the immune system. These findings have led to substantial interest for FXR as a therapeutic target and to the recent approval of an FXR agonist for treating primary biliary cholangitis as well as ongoing clinical trials for other liver diseases. Given that FXR biology is complex, including moderate expression in tissues outside of the enterohepatic circulation, temporal expression of isoforms, posttranscriptional modifications, and the existence of several other bile acid-responsive receptors such as TGR5, clinical application of FXR modulators warrants thorough understanding of its actions. Recent findings have demonstrated remarkable physiological effects of targeting FXR specifically in the intestine (iFXR), thereby avoiding systemic release of modulators. These include local effects such as improvement of intestinal barrier function and intestinal cholesterol turnover, as well as systemic effects such as improvements in glucose homeostasis, insulin sensitivity, and nonalcoholic fatty liver disease (NAFLD). Intriguingly, metabolic improvements have been observed with both an iFXR agonist that leads to production of enteric Fgf15 and increased energy expenditure in adipose tissues and antagonists by reducing systemic ceramide levels and hepatic glucose production. Here we review the recent findings on the role of intestinal FXR and its targeting in metabolic disease.

Keywords

Bile acids · Cholesterol · FGF19 · FXR · Glucose · Immunity · Intestine-selective · Metabolism

1 Introduction

The bile acid-activated farnesoid X receptor (FXR, NR1H4) is a major regulator of bile acid homeostasis and is, accordingly, most highly expressed in liver and intestine, i.e., the organs that physically constitute the enterohepatic circulation of bile acids. FXR belongs to the nuclear receptor family of ligand-activated transcription factors that are activated by hydrophobic molecules such as steroids, hormones, and fatty acids. Upon their activation these receptors control a wide array of processes in health and disease and, importantly, can be specifically targeted by pharmacological means. In fact, approximately 13% of all FDA-approved drugs target members of the nuclear receptor family (Overington et al. 2006). The identification of FXR as a bile acid-activated nuclear receptor in 1999 (Makishima et al. 1999; Parks et al. 1999; Wang et al. 1999), and the subsequent discovery of TGR5

(GPBAR1) as a bile acid-activated membrane-bound G protein-coupled receptor (Maruyama et al. 2002), has led to the identification of a series of novel physiological functions for bile acids that go far beyond their "classic" roles in the generation of bile flow and facilitating the uptake of dietary fats and fat-soluble vitamins (Hofmann and Hagey 2014; Kuipers et al. 2014). These novel functions in the regulation of glucose and lipid metabolism and in modulation of inflammation have raised substantial interest in designing therapeutic approaches that are based on interference with the bile acid-sensing machinery. Already in 2016, the FXR agonist obeticholic acid (OCA, Ocaliva also known as INT-747), an analogue of the natural FXR ligand chenodeoxycholic acid (CDCA), developed by Intercept Pharmaceuticals was the first treatment approved for primary biliary cholangitis (PBC) in 20 years and is currently under investigation in clinical trials for other liver diseases (Nevens et al. 2016). Fundamental studies on FXR have not only delineated a wide array of physiological functions for this nuclear receptor, but also pinpointed tissue-specific actions at its major sites of expression, the liver and intestine, as well as in other organs where expression is moderate yet with evident biological function. For instance, FXR activation in the kidney has nephroprotective effects (Herman-Edelstein et al. 2018), FXR modulates adipocyte differentiation in white adipose tissue (Abdelkarim et al. 2010), and it may even be involved in the etiology of depression through actions in the brain (Chen et al. 2018). In line with a "hormone-like function," bile acid pool size and composition, the rate of intestinal absorption of the individual bile acid species, and the efficiency of hepatic uptake of these intestine-derived bile acids during their enterohepatic cycling are increasingly recognized as important metabolic cues. For instance, some of the (immediate) beneficial metabolic effects of bariatric surgery have been attributed to altered bile acid metabolism (Spinelli et al. 2016; Albaugh et al. 2018), in particular via FXR-dependent changes in microbiome composition (Ryan et al. 2014). Yet, the exact contribution hereof and their underlying mechanisms, including a potential specific role of iFXR, remain to be elucidated.

FXR biology appears to be complex. Apart from its widespread organ and tissue expression, there are multiple FXR isoforms, posttranslational modifications, and regulatory cofactors that eventually all contribute to FXR activity. Therefore, meaningful pharmacological manipulation of FXR activity for therapeutic purposes is also complex but, at the same time, offers great opportunities for pursuit of novel strategies to develop selective modulators in a tissue- or function-specific manner. This has collectively been designated as the search for selective bile acid receptor modulators (SBARMs, as discussed by Massafra et al. 2018). This is not an easy task as, for instance, mice treated with the FXR ligand GW4064 showed binding of activated FXR to 6,345 binding sites on the genome in the liver upon ChipSeq analysis yet to 3,872 other sites in the intestine and to 1,449 joint sites in both organs. These sites were reported to change dramatically in obesity, illustrating both the complexity and the great potential of FXR modulation (Thomas et al. 2009; Lee et al. 2012).

The intestine is an attractive candidate for tissue-specific FXR modulation, as therapeutics can be administered orally, and, if bioavailability can be limited to the

gut, systemic release is avoided and side effects potentially prevented. At the same time, intestine-derived factors released upon treatment can exert systemic beneficial effects. Tissue-specific intestinal FXR (iFXR) modulators indeed appear to induce systemic metabolic improvements in obese mice, through actions in adipose tissue and liver (Fang et al. 2015; Pathak et al. 2018). However, these effects were paradoxically shown to occur with both activation and inhibition of iFXR, indicating that modulating the interplay of iFXR with its surroundings is not as straightforward as theoretically contemplated (Jiang et al. 2015a; Fang et al. 2015).

This review will focus on the physiological consequences of pharmacological modulation of iFXR. First, the characteristics and physiological function of endogenous iFXR will be outlined, starting with its role in modulation of bile acid homeostasis, since this aspect has to be taken into consideration when one plans to interfere with iFXR activity (Fig. 1). Recent findings on the physiological effects of selectively manipulating iFXR in pathophysiological settings will be discussed in the context of prospective applications of this promising therapeutic target.

1.1 Role of iFXR in Control of Bile Acid Metabolism

Because bile acids are "natural detergents", their hepatic synthesis rate, transport across cell membranes, and circulating pool size need to be tightly regulated, to ensure optimal concentrations in the intestinal lumen to facilitate nutrient absorption and, at the same time, prevent cytotoxicity at the sites where bile acids accumulate. Besides in hepatocytes (Goodwin et al. 2000), FXR in the distal ileum also contributes to the regulation of hepatic bile acid synthesis upon activation by circulating bile acids, thereby completing an effective negative feedback loop. In fact, under physiological conditions, signaling through iFXR appears to dominate de novo bile acid synthesis in rodents (Inagaki et al. 2005; Kim et al. 2007), and this regulation appears to be conserved in humans (Sjöberg et al. 2017). In short, the primary bile acids cholic acid (CA, 3α,7α,12α-trihydroxy-5β-cholan-24-oic acid) and CDCA (3α,7α-dihydroxy-5β-cholan-24-oic acid) are synthesized from cholesterol and conjugated to either taurine or glycine before their secretion into bile via the bile salt export pump (BSEP, ABCB11). In rodents the primary bile acids α- and β-muricholic acid (3α,6β,7α-trihydroxy-5β-cholan-24-oic acid and 3α,6β,7β-trihydroxy-5β-cholan-24-oic acid, respectively) are also synthesized. Upon secretion into the intestine, primary bile acids can be deconjugated by gut microbial bile salt hydrolases (BSH) and subsequently converted into secondary bile acids by bacterial 7α-dehydroxylases, leading to formation of deoxycholic acid (DCA) from CA and lithocholic acid (LCA) from CDCA. Likely due to the high prevailing bile acid concentrations, the microbial content is the lowest in the proximal part of the small intestine (10^3/g in duodenum) and gradually increases (10^7/g in ileum) toward the large intestine, where microbial counts are the highest (10^{12}/g in colon) (Mowat and Agace 2014). The capacity to deconjugate and dehydroxylate bile acids, consequently, increases along the intestinal tract. Bile acid deconjugation, which occurs largely in the distal part of the small intestine, is

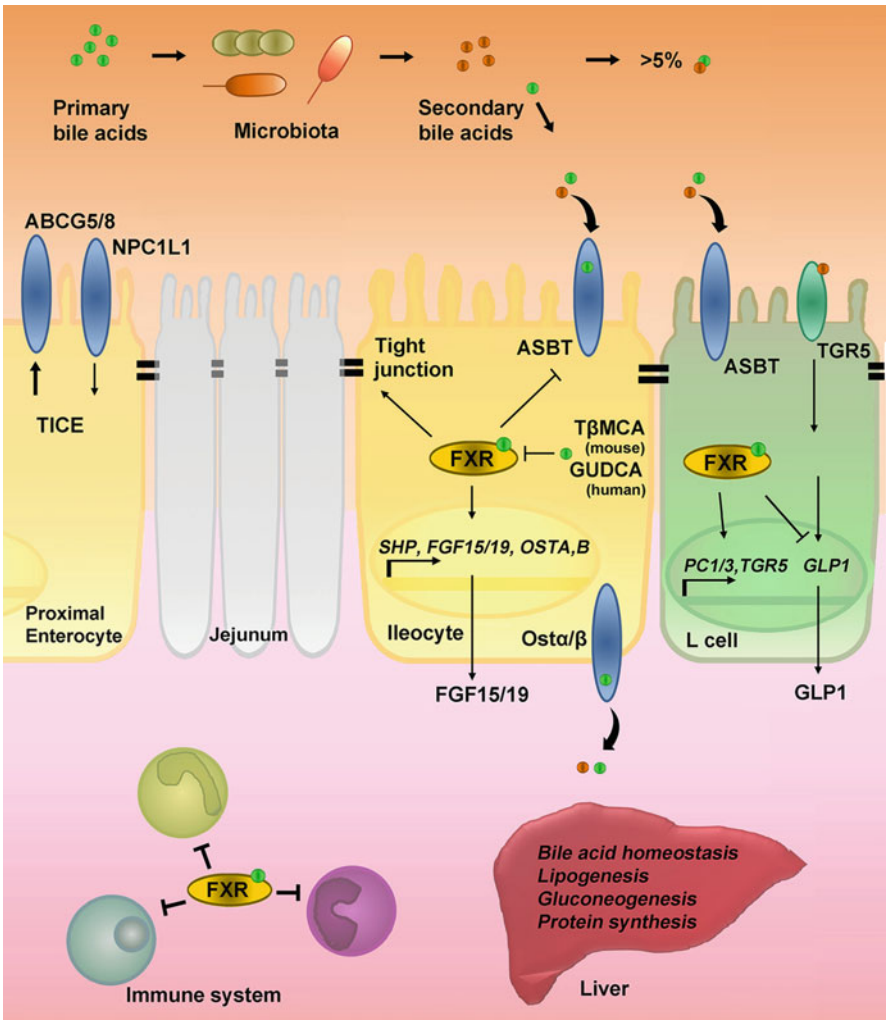

Fig. 1 Overview of intestinal FXR signaling. Following ingestion of a meal, conjugated bile acids are released from the gallbladder into the duodenum where they form mixed micelles that aid in the digestion of fats and fat-soluble vitamins. More distally, BAs can be deconjugated and converted into secondary bile acids by intestinal microbiota or/and transported into enterocytes via ASBT in the ileum. Upon bile acid uptake, iFXR can be activated, leading to transcription of *SHP*, *FGF15/19*, and *OSTA/B*, among others. Conjugated muricholic acids antagonize FXR activation in mice, and glycoursodeoxycholic acid (GUDCA) may do so in humans. At the basolateral side of the enterocytes, bile acids are transported by OSTα/β into portal blood. FGF15/19 is also secreted basolaterally, and both classes of compounds are transported to the liver. Upon reaching the liver, FGF15/19 inhibits hepatic bile acid synthesis. Activation of iFXR and FGF15/19 decreases hydrophobicity of the bile acid pool that stimulates transintestinal cholesterol excretion, predominantly upstream of the ileum. Bile acids stimulate TGR5 in the apical membrane of intestinal L cells, leading to release of glucagon-like peptide (GLP1), a process that appears to be modulated by FXR. Importantly, *GCG* expression, encoding preproglucagon and ultimately GLP1, is also suppressed by FXR. FXR activation furthermore maintains intestinal barrier function by stimulation of tight junction formation and suppressing inflammation in immune cells. Both GLP-1 and FGF15/19 have distinct systemic effects by activating their respective receptors in various organs

required for passive diffusion across the intestinal membrane. Active transport by the apical sodium-bile acid transporter (ASBT) is also confined to the ileum. This ecosystem ensures a sufficiently high bile acid concentration in the upper intestine to enable fat absorption. CDCA can be epimerized at the C7 position by some bacterial species to yield ursodeoxycholic acid (UDCA), a relatively hydrophilic bile acid (Fedorowski et al. 1979). Thus, there is a mutual interaction between the microbiome and bile acids, which might benefit bile acid-metabolizing strains (Friedman et al. 2018). Each bile acid species with its specific number and orientation of hydroxyl groups and, hence, hydrophobicity (Heuman 1989) differs in its ability to activate FXR (and TGR5). Therefore, local bile acid concentrations as well as composition will determine ensuing physiological responses. CDCA has been recognized as the most potent endogenous ligand of FXR, followed by DCA, LCA, and CA, whereas TGR5 is most potently activated by LCA, followed by DCA (Pathak et al. 2017). In contrast, TβMCA is a natural FXR antagonist in mice (Mueller et al. 2015; Sayin et al. 2013), while glycoursodeoxycholic acid (GUDCA) was recently reported to act as an FXR antagonist in humans (Sun et al. 2018a). LCA is the most hydrophobic bile acid species present in the adult human bile acid pool and is considered the most toxic (i.e., cholestatic), whereas the hydrophilic UDCA strongly stimulates bile flow (i.e., choleretic) and is being used in the treatment of cholestatic conditions such as PBC and intrahepatic cholestasis of pregnancy (Lefebvre et al. 2009; Cariello et al. 2018).

Bile acids are excreted from ileocytes (i.e., ileal enterocytes), into the blood by the basolaterally localized transporters OSTα/β and subsequently travel to the liver. After hepatic uptake by the basolateral transporter NTCP (SLC10A1), intracellular bile acids can activate hepatic FXR to regulate bile acid synthesis by inducing small heterodimer partner (SHP, NR0B2), which suppresses the expression of *CYP7A1* and *CYP8B1* (cholesterol 7 alpha-hydroxylase and sterol 12-alpha-hydroxylase respectively), encoding key enzymes in the conversion of cholesterol to bile acids (Lu et al. 2000; Goodwin et al. 2000). Importantly, iFXR induces production of fibroblast growth factor 19 (FGF19) in humans and Fgf15 in mice that also contributes to the regulation of hepatic bile acid synthesis (Kim et al. 2007). Composition, and hence hydrophobicity, of the bile acid pool is an important determinant of several physiological functions of circulating bile acids. This hydrophobicity is determined by the ratio in which the distinct primary bile acids are being formed as well as by their conversion into secondary species in the gut. For example, stimulation of iFXR in mice with the selective FXR agonist PX20606 leads to a very hydrophilic bile acid pool in mice, consisting almost exclusively of muricholic acids, that drives increased excretion of cholesterol from enterocytes into the intestinal lumen, mostly in the duodenum (described below, de Boer et al. 2017). Importantly, iFXR also contributes to control of (postprandial) gallbladder filling, through FGF15/19-induced relaxation of the gallbladder smooth muscle, hence modulating the dynamics of the enterohepatic circulation of bile acids (Choi et al. 2006).

Of note, the nuclear receptors pregnane X receptor (PXR; NR1I2), vitamin D receptor (VDR; Nr1I1), constitutive androstane receptor (CAR; NR1I3), as well as

the sphingosine 1-phosphate receptor 2 (S1PR2) are all also activated by bile acids (recently reviewed by Shapiro et al. 2018; Massafra et al. 2018). As activation of FXR modulates bile acid composition as well as bile acid concentrations in the various body compartments, manipulating FXR activity inevitably also affects the activities of the other bile acid-responsive receptors.

A large variation in bile acid composition is observed in the general human population (e.g., Luo et al. 2018), reflecting the interplay between bile acid sensors and factors such as lifestyle, diet, and the microbiome in the maintenance of bile acid homeostasis. Furthermore, various disease states are characterized by altered bile acid composition: this does not only concern liver diseases (Armstrong and Guo 2017; Trauner et al. 2017) but also cystic fibrosis (Bertolini et al. 2019) and metabolic diseases such as type 2 diabetes (e.g., Brufau et al. 2010). This widely varying "endogenous FXR activating capacity" has to be taken into account in the development of new therapeutic strategies based on interference with FXR activity rather than assuming that a one-size-fits-all procedure will be successful. Along the same line, it is evident that preclinical studies in murine models are of limited translational value because of the presence of large amounts of hydrophilic muricholates with antagonistic actions. Therefore mouse models with a humanized bile acid pool are urgently needed.

2 Intestinal FXR Isoforms and Posttranscriptional Regulation

The majority of intestinal bile acids is taken up via ASBT in the terminal ileum, leading to activation of FXR in ileocytes. However, FXR is expressed along the entire length of the small intestine as well as the colon (as deduced from human and mouse literature using Genevestigator analysis, data not shown, Zimmermann et al. 2004). Yet, the physiological role of FXR in the proximal part of the gastrointestinal tract, and its potential ligands, is still ill-defined. Next to these local distribution patterns, there are additional levels of regulation that may impact on physiological outcome of FXR activation in the various parts of the intestine. First, it is well known that multiple isoforms of FXR exist in humans and in rodents, with differences in spatiotemporal expression patterns, and that FXR is subject to posttranslational modification. These aspects may all contribute to fine-tuning of iFXR activity and will be further discussed in the next paragraphs.

2.1 Farnesoid X Receptor Isoforms

Human FXRα (or NR1H4) is expressed from a single gene locus located on chromosome 12 (12q23.1) and murine FXR on chromosome 10 (10:89454234–89533585). Differential promoter regulation and alternative splicing result in four different isoforms (FXRα1, FXRα2, FXRα3, and FXRα4) both in humans and in mice (Zhang et al. 2003; Huber et al. 2002). Two promoters, present in front of exon 1 and 3, induce either expression of FXRα1,2 or α3,4 (Fig. 2a). A point of caution,

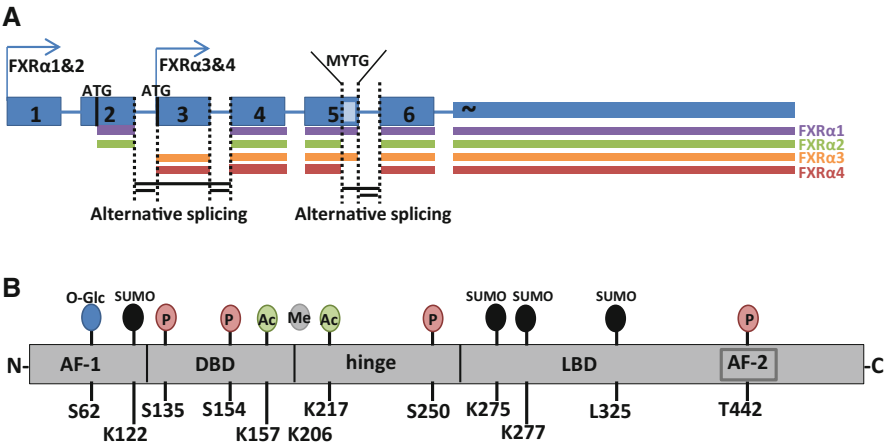

Fig. 2 (**a**) The farnesoid X receptor gene structure and predicted isoforms. (**b**) FXR protein domains and reported sites for modifications by acetylation, phosphorylation, SUMOylation, and O-GlcNAcylation as detailed in the text

generally overlooked, is that public databases (NCBI, ENSEMBL) only contain three RefSeq transcripts of mouse FXR, missing the α2 isoform. Moreover two additional splice variants of the human α1 isoform are reported that encode the same protein. Besides FXRα, mice have a functional pseudogene FXRβ, which appears nonfunctional in primates, and that is activated by the cholesterol synthesis intermediate lanosterol (Otte et al. 2003). FXRβ will not be considered in this chapter.

Expression of human *FXR* is highest in ileum, followed by liver, duodenum, kidney, and colon (Vaquero et al. 2013). Genevestigator database analysis showed a similar order in mice, with the exception of a relatively high *Fxr* expression in the kidney, comparable to that in the liver (data not shown, Zimmermann et al. 2004; Boesjes et al. 2014). FXRα1–4 isoforms were reported to be equally expressed in the liver, while FXRα3 and FXRα4 predominate in duodenum, jejunum, and ileum (Zhang et al. 2003; Boesjes et al. 2014). Regulation of FXR target genes is reported mostly in an isoform-independent manner; however, some genes (e.g., *IBABP*) appear to be more responsive to FXRα2/FXRα4 than to FXRα1/FXRα3, due to the lack of the additional amino acids next to the DNA-binding domain (MYTG motif) (Zhang et al. 2003). The lack of this MYTG motif in FXRα2 and FXRα4 in general results in higher transcriptional activity (Gray and Squires 2015). The presence of the tyrosine phosphorylation site in the MYTG motif was suggested to be responsible for differences in variant activity (Gray and Squires 2015). Whether MYTG is increasing or decreasing the activity of FXR seems to be species-dependent. In mice, hepatic FXRα2 was proven to be more effective than FXRα4 in reducing HDL- and VLDL-cholesterol levels and in switching hydrophobicity of the bile acid pool due to differential regulation of *Cyp8b1* expression. In addition, hepatic FXRα2 caused an increased fecal neutral sterol excretion without affecting intestinal cholesterol absorption when compared to hepatic FXRα4 (Boesjes et al.

2014). Furthermore, a cell-specific pattern of FXR isoforms seems to determine the tissue sensitivity to FXR agonists, which may also be specific for different target genes (Zhang et al. 2003; Vaquero et al. 2013). Selective effects of the different isoforms specifically on iFXR targets have to the best of our knowledge not been reported.

The transcription factor GATA binding protein 4 (GATA4) suppresses bile acid metabolism-related genes in the jejunum: this regulator of intestinal development defines jejunal versus ileal identity (Walker et al. 2014; Thompson et al. 2017). Disruption of the microbiota with antibiotics resulted in jejunal expression of *Asbt* in a *Gata4*-dependent manner that confines *Asbt* expression to the ileal part of the intestine (Out et al. 2015). Bile acid-mediated activation of FXR in the small intestine therefore appears to be mainly restricted to terminal ileum by an interaction between GATA4 and the microbiota (Out et al. 2015).

2.2 Posttranscriptional Regulation of FXR by Phosphorylation, Acetylation, O-GlcNAcylation, or SUMOylation

Posttranslational regulation of FXR has been reported and has been related to various metabolic changes. These modulations can have both repressing and activating effects on FXR function (summarized in Fig. 2b). While most of these interactions with protein-modifying enzymes have been described for hepatic FXR, these enzymes are also present in the intestine, and they are therefore likely to act accordingly in enterocytes. In the next sections, we will discuss the different posttranslational modifications of FXR that need to be taken into account when studying the impact of pharmacological iFXR modulation, particularly in metabolic disease states.

Protein kinase C (PKC) has been shown to phosphorylate FXR at serine (S)135 and S154 in the DNA-binding domain, leading to recruitment of coactivator PGC1α and enhanced FXR transcriptional activity (Zhang et al. 2004; Gineste et al. 2008). In contrast, phosphorylation of S250 by AMPK, one of the main sensors of cellular energy levels, leads to repression of FXR, which, when left uncontrolled, leads to bile acid accumulation and hepatic injury (Lien et al. 2014; Becares et al. 2017). Phosphorylation of FXR by PKC ζ that is stimulated by ATP8B1 was shown to be one of the determinants of translocation to the nucleus (Frankenberg et al. 2008). Nuclear translocation was later shown to apply to all isoforms of FXR in a tissue- and species-independent manner (Vaquero et al. 2013). As a result, probably due to insufficient capacity of BSEP, accumulation of hepatic bile acids occurs when the phospholipid-transporting ATPase ATP8B1 does not function properly. Mutations in ATP8B1 cause progressive familial intrahepatic cholestasis type 1 (PFIC1, Bull et al. 1998). As extrahepatic manifestations of mutations in *ATP8B1* occur as well, including diarrhea that often does not resolve after liver transplantation, a similar regulation of FXR activity by ATP8B1/PKC-induced phosphorylation may occur in enterocytes. However, *ATP8B1* encodes for a phospholipid flippase, which may primarily affect the endothelial membrane and perhaps have secondary effects on

FXR. Yet, regulation of apical localization of proteins in endothelial cells by ATP8B1 was shown to be independent from its flippase activity (Verhulst et al. 2010). Direct evidence of FXR phosphorylation in the intestine has not been reported (Frankenberg et al. 2008).

FXR was shown to control expression of miR-34a and its target (among others) sirtuin 1 (SIRT1), a mediator of the beneficial effects of caloric restriction in rodents (Lee and Kemper 2010). The activity of several transcription factors involved in regulation of metabolic genes, including FXR, can in turn be altered by SIRT1-mediated deacetylation. Interestingly acetylated FXR levels are remarkably increased in fatty livers of obese mice, suggesting a decreased activity of SIRT1. Indeed, administration of SIRT1 agonists to these obese mice decreased FXR acetylation and had beneficial metabolic effects (Kemper et al. 2009). In addition, both acetylation of K157 and K217 were shown to stabilize FXR, coinciding with a decreased capability to heterodimerize with retinoid X receptor (RXR) (Kemper et al. 2009).

Increased acetylation of FXR at K217 in obese mice was shown to be associated with inhibition of SUMO2 modification at the K277 position, resulting in increased hepatic inflammation and metabolic dysfunction (Kim et al. 2014). In addition, SUMOylation of K122 and K275 leads to decreased recruitment of FXR to target gene promotors and increased interaction with nuclear factor-kappa beta (NF-kB) (Balasubramaniyan et al. 2013). Recently, another human SUMOylation site was identified (i.e., L325) in an FXR domain that is responsible for transcriptional coactivation. Posttranslational modification of this site was required to achieve efficient ligand activation (Bilodeau et al. 2017). Whereas posttranslational modifications have been primarily reported for hepatic FXR, remarkably little is known about acetylation and SUMOylation of iFXR in the intestine, i.e., in an organ with high metabolic rate.

In liver, O-GlcNAcylation of FXR (S62 in the AF-1 domain) occurs in response to glucose and was shown to increase FXR protein levels and transcriptional activity (Berrabah et al. 2014). Pathophysiology in relation to FXR O-GlcNAcylation has not yet been reported, but it has been suggested that this modification might affect FXR-mediated control on bile acid production (Benhamed et al. 2015). Additionally, in vitro methylation of FXR was reported in hepatocytes on K206 (Balasubramaniyan et al. 2012), and in a large proteomic analysis, ubiquitylation of hepatic FXR was identified as well (Wagner et al. 2012). As conjugated and unconjugated BAs seem to differentially activate FXR in different cell lines, an as yet undefined posttranscriptional modification of FXR or the recruitment of different coactivators to the transcription complex was proposed (Vaquero et al. 2013). Thus, it seems that conjugated BAs require an additional cellular mechanism to activate FXR, which, although the exact mechanism remains ill-defined, might have physiological implication along the length of the small intestine where unconjugated bile acids can be taken up proximally by passive diffusion and conjugated ones only in the ileum by active ASBT-mediated transport.

2.3 FXR in Development and Aging

An intriguing yet poorly explored aspect of FXR biology concerns its potential role(s) during the different phases of life. It is well established that bile acid metabolism in the fetus and newborn shows specific features, in part due to the development of the newborn's microbiome and in part dependent on the developmental pattern in the bile acid synthesis cascade, i.e., during the late fetal and early postnatal phase, a phase of physiologic cholestasis may occur that gives rise to increased ligand availability (Suchy et al. 1981; Stahl et al. 1993; Hill et al. 2017). Yet, surprisingly little is known about expression and activity of FXR during this period of continuous adaptive change. As a first step to gain a basic understanding of this period that is crucial for metabolic homeostasis later in life, we have performed a database search of iFxr expression in the intestine during the mouse life cycle (Genevestigator, Zimmermann et al. 2004), which revealed very high expression levels in utero that decrease somewhat after birth and remain relatively constant throughout life (Fig. 3a). To further specify iFxr expression in early life that was only

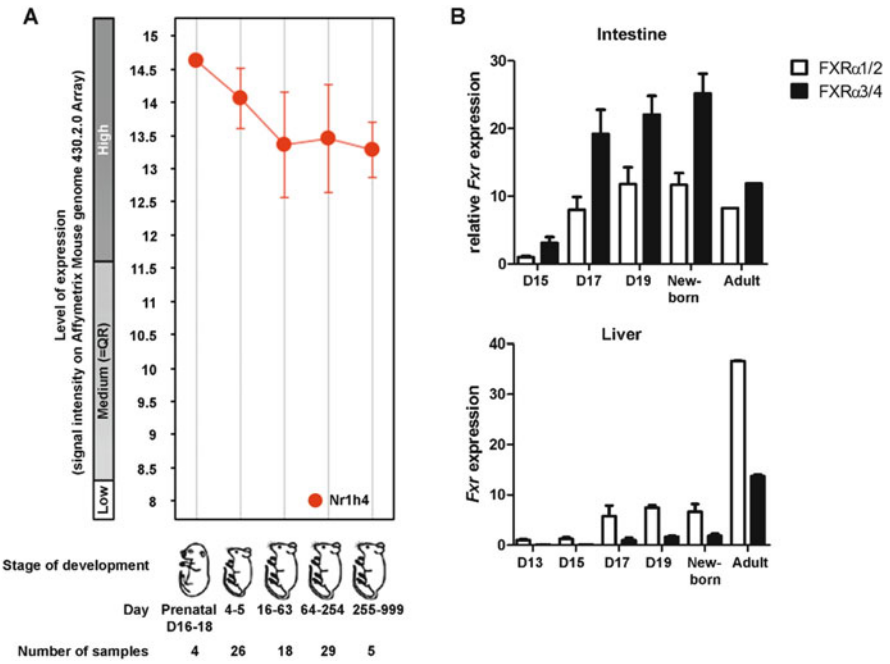

Fig. 3 (**a**) Genevestigator analysis of intestinal Fxr (Nr1h4) expression during the murine life course, showing highest expression prior to birth. (**b**) Intestinal and hepatic Fxr isoform expression in utero, showing increasing expression from embryonic day 15 until birth with highest expression of Fxr α3/α4 in the intestine and predominating expression of the α1/α2 isoforms in the liver that is highest in adult mice

represented by a single time point in the database analysis, we determined mRNA levels of FXR isoforms α1/2 and α3/4 in intestine and livers of developing mouse embryos and compared these to adult levels. Figure 3b shows that in intestine isoforms α3/4 dramatically increase from embryonic day 15 after pregnancy to day 19, while expression of α1/2 lags behind. Fetal expression in the intestine appeared to be higher than in adults. In contrast, in the fetal liver, isoforms α1/2 dominate over α3/4 and increase from day 15 until birth, but hepatic expression appears to be lower in the fetus than in the adult. It should be emphasized that the physiological relevance hereof awaits further study.

Postnatally, various metabolic changes occur that have been associated with age-related development of metabolic diseases. For example, decreased conversion of cholesterol into bile acids potentially contributes to risk of cardiovascular disease which is decreased in aging (Charach et al. 2017), implying changes in bile acid receptor activity (Uranga and Keller 2010; Joyce and Gahan 2016). Indeed, it has been shown that *FXR* expression in kidney and liver is decreased in aged mice (Xiong et al. 2014; Wang et al. 2017), whereas our analysis did not show this for iFxr (Fig. 3b). In contrast, aging of both whole-body and liver-specific *Fxr/Shp* double knockout mice showed reversal of body weight gain, adiposity, and glucose/insulin tolerance associated with aging (Kim et al. 2017). Intestine-specific FXR reactivation restores bile acid homeostasis in young and aged mice that lack FXR, which protects them from hepatocellular carcinoma development that is associated with age-related changes in bile acid metabolism (Degirolamo et al. 2015). Obesity has also shown to decrease *Fxr* expression in adipose tissue (Cariou et al. 2006), and diabetes suppresses hepatic *Fxr* expression (Duran-Sandoval et al. 2004). In contrast, iFXR expression levels are not susceptible to obesity in mice and rats (Chen et al. 2010; Stenman et al. 2012).

3 Biology of Intestinal FXR

Understanding the physiological functions of iFXR allows prediction of the relevant metabolic processes that can be targeted by modulating its activity as well as of the potential side effects that could arise. Beneficial and adverse effects may both stem from either direct local FXR activity or from its transduction to other sites of the body by mediators such as hormones and growth factors or specific lipid species but also through adaptations in bile acid metabolism that lead to changes in bile acid pool composition, pool size, and/or cycling frequency.

3.1 iFXR, Intestinal Barrier Function, and Immunity

Composition of the microbiome, intestinal immune function, and integrity of the intestinal barrier are the major lines of defense at the intestinal surface, the largest surface of our body that is exposed to the outside world, and are all regulated by iFXR. Gut dysbiosis includes all three aspects and is a key feature of obesity and

associated metabolic dysfunction, although we are only beginning to understand the mechanisms underlying these complex relationships (Teixeira et al. 2012; Sun et al. 2018b). It is well known that bile acids exert direct antibacterial effects in the small intestine, likely one of the reasons why bacterial numbers are low in the upper GI tract where bile acids enter (Binder et al. 1975; Ding et al. 1993). Vice versa, the microbiome also interacts with bile acid metabolism and iFXR signaling. Feeding the antioxidant tempol to mice inhibits iFXR signaling by altering the microbiome leading to an accumulation of intestinal tauro-β-muricholic acid (T-β-MCA), a potent FXR antagonist (Li et al. 2013). Importantly, iFXR was found to mediate the anti-obesity effects of tempol in high-fat diet-fed mice. Likewise, it was recently postulated that metformin, the first-line medication in the treatment of diabetes, acts in part by reducing numbers of *Bacteroides fragilis*, a bacterial species with bile acid-deconjugating activity, leading particularly to increased abundance of the FXR antagonist glycoursodeoxycholic acid (GUDCA) in humans. On the basis of an additional series of mouse experiments, showing that high-fat diet-fed mice colonized with *Bacteroides fragilis* showed more severe glucose intolerance and less sensitivity to the benefits of metformin treatment, it was concluded that metformin acts at least in part through the *B. fragilis*-GUDCA-iFXR axis to improve metabolic health (Sun et al. 2018a). Administration of the FXR agonist GW4064 abrogated small intestinal bacterial overgrowth and decreased bile duct ligation-induced intestinal permeability and inflammation in mice (Inagaki et al. 2006; Verbeke et al. 2015). *Fxr*-deficient mice are much more susceptible to these pathologies, i.e., tenfold higher aerobic bacterial counts were observed in lymph nodes in bile duct-ligated *Fxr*-deficient mice and even with a sham operation the lymph nodes of these mice already contained more aerobic bacteria than those of control animals. In accordance, the epithelial barrier of *Fxr*-deficient mice appeared to be deteriorated (Inagaki et al. 2006). Positive effects of iFXR activation are possibly in part mediated by the FXR targets inducible nitric oxide synthase (iNOS) and IL18 (Verbeke et al. 2015). A genetic variant in human *FXR* is associated with inflammatory bowel disease (Attinkara et al. 2012), and FXR activity in the ileum appears to be decreased in patients with Crohn's disease (Nijmeijer et al. 2011). In a chemically induced colitis mouse model, the inflammatory response could be suppressed by the FXR agonist OCA in a *Fxr*-dependent manner (Vavassori et al. 2009). Furthermore, FXR agonism was shown to suppress expression of Toll-like receptor 4 (TLR4) and NF-κB regulated pro-inflammatory genes in colon macrophages, as also observed in liver (Vavassori et al. 2009; Wang et al. 2008). Similar outcomes of FXR agonism have been observed in a mouse model of intestinal ischemia and could therefore be relevant for pathologies in which aberrant gut-liver axis signaling plays a role (van Erpecum and Schaap 2015; Ceulemans et al. 2017). Indeed, the anti-inflammatory actions of FXR extend well beyond the intestine, for instance, also in blood leukocytes (Gadaleta et al. 2011a). Moreover, OCA treatment has similar effects in lipopolysaccharide-induced lung and kidney injury in mice, suggesting a general mechanism of action (Gai et al. 2016; Fei et al. 2019). Indeed, direct interaction of FXR with NF-kB signaling has been identified in many cell types as the underlying mechanism for NF-kB

transrepression. This interaction may also explain reported bidirectional effects, as inflammation can in turn inhibit FXR activity, one of many ways by which metabolism and inflammation are intertwined (Gadaleta et al. 2011b; Verbeke et al. 2016). For instance, activation of membrane-bound TLR4 downregulates FXR expression in human monocytes, whereas intracellular TLRs such as TLR9 upregulate *Fxr* expression in human monocytes and in enterocytes of mice with colitis (Renga et al. 2013).

3.2 Modulation of Intestinal Cholesterol Metabolism by iFXR

The intestine plays an important role in the maintenance of cholesterol homeostasis by mediating its absorption from bile and diet as well as by active export, a process referred to as transintestinal cholesterol export (TICE, Temel and Brown 2015; de Boer et al. 2018). The majority of (dietary and biliary) cholesterol is absorbed proximally in the small intestine, and the presence of bile acids is an absolute requirement for absorption to occur (Voshol et al. 2001). The hydrophobicity of the intestinal bile acids impacts on lipid absorption in general, including cholesterol, due to the greater potency of hydrophobic bile acid species to incorporate lipid-soluble nutrients within the mixed micelles that are required for transport. Whether or not differences in bile acid pool composition contribute to the large variability in fractional cholesterol absorption between human subjects has remained unresolved so far (Bosner et al. 1999).

Besides absorption, the intestine can also regulate cholesterol turnover via TICE, a process that is subject to different modes of control. This route accounts for about 30% of fecal cholesterol loss in chow-fed mice as well as in humans (recently reviewed by de Boer et al. 2018). Inhibition of cholesterol absorption through inhibition of the cholesterol import transporter NPC1L1 by ezetimibe also increases the TICE pathway (Nakano et al. 2016; Jakulj et al. 2016), conceivably by preventing reuptake of cholesterol that is effluxed into the intestinal lumen by ABCG5/8 in enterocytes, leading to an increased net removal of cholesterol (de Boer et al. 2018). Interestingly, stimulation of FXR by the nonsteroidal agonist PX20606 (Abel et al. 2010) induced a strong increase of cholesterol removal via the TICE pathway in mice that was dependent on *iFxr*, i.e., an effect that was maintained in mice expressing *Fxr* only in the intestine (de Boer et al. 2017). Moreover, the effect on fecal cholesterol loss was completely additive to that of ezetimibe, causing extremely augmented cholesterol turnover upon combined treatment. Mechanistic experiments indicated a critical role for bile acid pool composition in the stimulation of TICE. In mice, iFXR activation and FGF19 administration were shown to shift the balance of the bile acid pool toward hydrophilic bile acid species, i.e., the muricholic acids, due to a relatively strong repression of *Cyp8b1* expression (de Boer et al. 2017). These hydrophilic bile acid species may stimulate the activity of ABCG5/8 and hence cholesterol export into the lumen (Berge et al. 2000; Bonamassa and Moschetta 2013; de Boer et al. 2017). However, additional iFXR-dependent mechanisms may be operational as well, since fecal cholesterol excretion was only

modestly increased in high-fat diet-fed *Cyp8b1*-deficient mice that show a similar hydrophilic bile acid pool composition (Bonde et al. 2016). Whether or not therapeutic targeting of iFXR may be beneficial for treatment of hypercholesterolemia to prevent cardiovascular disease in humans remains to be established. Stimulation of TICE via modulation of bile acid pool composition is not expected to be conserved in humans based on the marked differences in pool composition between humans and mice. In fact, in humans *CYP8B1* repression will theoretically lead to a CDCA-dominated, hydrophobic bile acid pool and hence more effective cholesterol absorption and less TICE.

An additional layer of complexity in the role of iFXR in control of cholesterol absorption was recently described by Kim et al. (2018), showing that iFXR-mediated release of Fgf15 from the murine ileum signals to the proximal small intestine to repress expression of *Npc1l1* and, thereby, cholesterol absorption. In a series of experiments, exploiting *Shp*-deficient and *Fgf15*-deficient mice, data were presented to indicate that ileum-derived Fgf15 as well as i.v. injected FGF19 led to phosphorylation of SHP which suppressed activity of sterol regulatory transcription factor 2 (SREBPF2) that regulates sterol-activated transcription of *Npc1l1* in enterocytes of the upper small intestine. Thus, FGF15/19 may not only modulate the cholesterol-solubilizing capacity of mixed micelles in the lumen of the small intestine but also the expression of the cholesterol transporter at the apical membrane of the enterocytes. Consequently, the overall effect of iFXR activation and Fgf15 release in mice is a strong acceleration of cholesterol turnover by stimulation of TICE and reduction of fractional cholesterol absorption, the latter by a dual mode of action. Whether this also applies to humans, with a more hydrophobic bile acid pool, remains to be established. At the same time, obviously, suppression of hepatic bile acid synthesis, which comprises a very important pathway for cholesterol turnover in humans as well as rodents, will impair (hepatic) cholesterol turnover. This may underlie (part of) the reported LDL elevations upon pharmacological FXR activation (Pencek et al. 2016; Neuschwander-Tetri et al. 2015).

3.3 Role of iFXR in Control of Glucose Metabolism

In the past decade, several studies have reported a role for FXR in control of glucose metabolism, in most cases employing whole-body *Fxr* knockout mouse models and/or systemically acting FXR modulators. Yet, some interesting features concerning specific roles of iFXR in control of intestinal and whole-body glucose metabolism with potential therapeutic potential have also been reported. To the best of our knowledge, only a single report so far has demonstrated a role for iFXR, particularly in the proximal small intestine, in the kinetics of glucose absorption. Employing different stable isotopically labeled glucose tracers and compartment modelling, van Dijk et al. (2009) demonstrated that absorption of glucose is delayed in whole-body *Fxr*-deficient mice, due to an increased flux of glucose molecules through the glucose-6-phosphate pool in enterocytes before entering the bloodstream. Thus, while uptake of glucose by enterocytes was similar in wild-type and

Fxr-deficient mice, the residence time of glucose molecules within the enterocytes was longer in *Fxr*-deficient mice due to enhanced hexokinase 1 and 2 (Hk1/2)-mediated phosphorylation. Glucose-6-phosphate subsequently requires dephosphorylation by glucose-6-phosphatase before glucose can be released via this indirect pathway into the blood. Indeed, the expression of *Hk1* and *Hk2* was sixfold higher in the proximal part of the small intestine of *Fxr*-deficient mice compared to the controls while all other transporters and enzymes involved in glucose handling were unaffected. Whether or not the kinetics of intestinal glucose absorption is modulated upon pharmacological FXR activation remains to be established.

Effects of a deficiency in *iFXR* on systemic glucose metabolism have been studied in more detail. *Fxr* and *Tgr5* are co-expressed in enteroendocrine L cells, and activation of TGR5 by luminal bile acids regulates intestinal production of the incretin glucagon-like peptide 1 (GLP-1) that in turn stimulates pancreatic insulin secretion and glucose homeostasis (Pathak et al. 2017; Thomas et al. 2009). GLP-1 and gastric inhibitory peptide (GIP) are the most important incretins and the basis of incretin mimetic therapies for type 2 diabetes, as well as dipeptidyl peptidase-4 (DPP-4) inhibitors that prevent the degradation of endogenous incretins (reviewed in Ahrén 2012). FXR appears to modulate *Tgr5* expression, stimulating GLP-1, yet FXR also suppresses preproglucagon, encoding GLP-1 (Trabelsi et al. 2015). A dynamic interaction between FXR and TGR5 was subsequently proposed to potentiate GLP-1 action in the control of glycemic control (Kim and Fang 2018).

Low serum FGF19 levels were associated with high fasting plasma glucose levels and type 2 diabetes (Fang et al. 2013), and FGF19 action has been implicated in hepatic glucose metabolism (Potthoff et al. 2011). However, long-term central effects of FGF19 have also been shown to contribute to glucose lowering upon a single intracerebroventricular injection (Fu et al. 2004; Morton et al. 2013). Interestingly, a role for the central nervous system was recently confirmed by showing its dependence on β-Klotho specifically in the nervous system, while the acute glucose-lowering effects were shown to depend on β-Klotho in adipose tissue (Lan et al. 2017).

Paradoxically, there is recent evidence supporting that inhibition, rather than activation, of *iFxr* improves glucose metabolism during metabolic disease conditions. Mice with iFxr deficiency are protected from diet-induced obesity and insulin resistance (Li et al. 2013; Jiang et al. 2015a; Xie et al. 2017). In addition, obese *iFxr*-deficient mice treated with tempol, which reduces microbiota species with high BSH activity and therefore increases levels of the FXR antagonist T-β-MCA, lacked the observed decrease in blood glucose and insulin levels that occur in control animals, indicating the significance of iFXR for this approach (Li et al. 2013). These effects were associated with decreased ceramide levels and could be reversed by ceramide administration (Jiang et al. 2015b). The translational potential of these studies, however, remains uncertain, as humans do not produce muricholic acids. To overcome this translational issue, mice with a humanized bile acid pool have recently been generated by deleting the *Cyp2C* cluster (Takahashi et al. 2016).

3.4 iFXR and Energy Metabolism

The potential of iFXR to control energy homeostasis has attracted strong interest in the development of therapeutics in the management of various metabolic syndrome-associated morbidities. FGF15/19 is an important mediator of these effects. Transgenic mice constitutively expressing *FGF19* in muscle have increased brown adipose tissue size and energy expenditure resulting in reduced body weight upon high-fat diet feeding. FGF19 treatment of obese mice has similar effects (Tomlinson et al. 2002; Fu et al. 2004). Despite a low sequence similarity between murine *Fgf15* and human *FGF19*, both genes are syntenic, and their biological function in the regulation of bile acid homeostasis is well conserved. As FGF19 protein has a stability superior to Fgf15, transgenic expression and treatments have almost exclusively been performed with FGF19. The interaction between iFXR and the microbiome is also one of the major routes by which microbiota impact host metabolism and therefore represents an indirect approach to target iFXR (Sayin et al. 2013; Degirolamo et al. 2014; Sun et al. 2017). Besides beneficial metabolic effects, it was also shown that microbiota-induced obesity in mice requires *iFxr* (Li et al. 2013; Zhang et al. 2016; Parséus et al. 2017).

Next to suppression of bile acid synthesis, many other hepatic processes are under iFXR control as well, depending for a large part on FGF15/19 signaling.

3.5 Hepatic Metabolism and iFXR

Hepatic activation of FXR exerts beneficial effects in nonalcoholic fatty liver disease (NAFLD) by repressing de novo lipogenesis and promoting fatty acid oxidation and VLDL clearance (Zhang and Edwards 2008). Clinical trials with OCA have shown initial improvements in histological features of NASH (Neuschwander-Tetri et al. 2015), although more studies are needed to assess long-term efficacy and safety. Release of FGF15/19 by iFXR activation and subsequent binding to fibroblast growth factor receptor 4 (FGFR4) and its co-receptor β-Klotho on hepatocytes initiates a signaling cascade that not only suppresses bile acid synthesis but also gluconeogenesis and promotes protein synthesis (Kir et al. 2011). Furthermore, FGF15/19 decreases hepatic lipogenesis (Fuchs et al. 2016) and could indirectly stimulate mitochondrial fatty acid oxidation (Tomlinson et al. 2002; Fu et al. 2004). Despite these premises, there is also evidence supporting that iFXR inhibition rather than activation could improve NAFLD. Mice lacking *iFXR* expression remain lean during a high-fat diet challenge, possibly as a consequence of lower intestinal production of ceramides, resulting in the downregulation of *Srebp1c* and thus decreased lipogenesis (Jiang et al. 2015a). Similar results were obtained with pharmacological ASBT inhibition that reduces iFXR ligand availability and paradoxically also with inhibition of iFXR (described below). A successful phase II clinical trial was recently reported in NASH patients with NGM282 an FGF19 analogue (Harrison et al. 2018). FGF19 administration in mice also represses hepatic gluconeogenesis via cAMP response element-binding protein (CREB) (Potthoff

et al. 2011). These effects of FGF19 seem analogous to the actions of insulin; however, they occur independently and through different pathways (Kir et al. 2011).

3.6 Temporal Regulation of iFXR and Oncogenic Effects

Besides studies on iFXR that are based on tissue-specific receptor deficiency, tissue-specific constitutively active overexpression has also been applied, albeit mostly to evaluate effects on tumorigenesis. These studies showed that transgenic mice with an intestine-specific constitutive expression of *Fxr* (iVP16FXR) were protected from hepatocellular carcinoma (Degirolamo et al. 2015). Intestinal FXR targets such as *Fgf15*, *Shp*, and *Ostα/β* were affected in iVP16FXR mice, and a 30% reduction in bile acid pool was observed, as well as both decreased inflammation and tissue damage in intestine and in liver in models of cholestasis (Modica et al. 2012). Metabolic effects from this constitutively active model are however difficult to translate, as endogenous FXR activity oscillates and occurs predominantly postprandially. We and others identified iFXR as a modulator of hepatic diurnal rhythm through FXR-FGF15 signaling (Stroeve et al. 2010), and plasma Fgf15 also showed an FXR-dependent circadian rhythm (Katafuchi et al. 2015). In line with a physiological function in the postprandial phase is the observation of suppressive actions of FXR on the intracellular quality control mechanism, autophagy, that is mostly active in the fasted state (Lee et al. 2014; Seok et al. 2014). As autophagy is a pathway that degrades superfluous components in the cell, both inactive and overly active autophagies are detrimental. Therefore, autophagy is highly interlinked with the circadian clock, and strict temporal regulation is warranted (Toledo et al. 2018). FGF19 also transduces a suppressive signal on autophagy in the liver (Byun et al. 2017). Moreover, regulation by FXR of *Sqstm1* encoding P62, an important factor in selective autophagy, occurred especially in the intestine (Williams et al. 2012). Likewise, the second proteolytic quality control mechanism, the unfolded protein response (UPR) that is known to respond to a meal (Liu et al. 2018), also has a cytoprotective function if kept at bay. Sustained activation of the UPR, however, for example, during chronic ER stress, can lead to disease (Fu et al. 2012) and aging may downregulate hepatic *FXR* due to sustained ER stress, leading to hepatic steatosis (Xiong et al. 2014). For instance, constitutive activity of ATF6, one of the three UPR sensors in the intestine, leads to loss of intestinal barrier function and microbiota-dependent tumorigenesis (Coleman et al. 2018). Albeit less established, recent findings have also identified hepatic FXR as a regulator of the other two UPR sensors PERK and IRE1α/XBP1 (Liu et al. 2018; Han et al. 2018). These interactions suggest that a tight temporal regulation of iFXR is required to maintain cellular homeostasis, as both over- and underrepresentation of iFXR output has been implicated in tumor development.

3.6.1 Oncogenic Potential of iFXR Modulation

As FGF15/19 is an endocrine member of the FGF family that has been implicated in the control of cellular proliferation and development, modulating iFXR may come

with some potential carcinogenic risks. Indeed, *Fgf15* overexpression induces hepatocyte proliferation in mice, independently of hepatic bile acid levels, both with and without previous partial hepatectomy (Kong et al. 2012). FGF19 transgenic mice develop hepatocellular carcinomas that is dependent of FGFR4 (Nicholes et al. 2002; French et al. 2012), and as a result *FGF19* has been coined an oncogene (Cui et al. 2018). Interestingly, FGF19 but not Fgf15 induces hepatocellular carcinoma at supraphysiological levels in mouse models of metabolic syndrome, perhaps due to the low stability of the latter (Zhou et al. 2017). In humans, increased *FGF19* expression was found in hepatocellular carcinoma and correlated with tumor progression (Miura et al. 2012). In contrast, in mouse colon, *Fgf15* deficiency resulted in increased cellular proliferation, crypt-villus length, and advanced neoplasia (Cheng et al. 2018). Similar effects were also observed in *Asbt*-deficient mice, possibly due to increased TGR5 signaling (Thulesen 2004; Raufman et al. 2015). Additionally, *Fxr* deficiency promotes intestinal tumor development in different models of colon cancer, by increasing cell proliferation and inflammation (Modica et al. 2008; Maran et al. 2009). In line with a metabolic rheostat function for FXR, potential carcinogenic risks exist upon unbalanced activation as well as deactivation that must be taken into account when designing drugs that target iFXR.

4 Approaches for Intestine-Specific Targeting of FXR

As interference with bile acid-responsive receptors can invoke potent physiological responses, generation of novel modulators with selectivity for a single receptor or even a subset of its target genes has gained substantial interest (Massafra et al. 2018). Studies reporting pharmacological approaches to either activate or inhibit iFXR and the physiological consequences will be discussed below.

4.1 Selective Activation of iFXR: Fexaramine

The observation that the FXR agonist fexaramine (Fex) is poorly absorbed by the intestine but still mediates potent activation of iFXR opened up an avenue to specifically target iFXR (Fang et al. 2015). Obese mice treated for 5 weeks showed reduced fat mass and metabolic improvement as well as reduced systemic inflammation. These beneficial effects were attributed to increased energy expenditure due to brown adipose tissue activity, browning of white adipose tissue, and a shift in bile acid composition favorable for TGR5 activity. Indeed, in line with a role for TGR5 in activating brown adipose tissue (Watanabe et al. 2006), the effects were partially dependent on whole-body *Tgr5* (Fang et al. 2015). These findings were recently confirmed in obese leptin receptor-deficient *db/db* mice and extended by showing a change in microbiota composition upon Fex treatment and reversal of the Fex-induced metabolic improvement after antibiotic treatment (Pathak et al. 2018).

4.2 Selective Inhibition of iFXR

Similar to the situation in obese mice treated with tempol, which reduces microbial conversion of the FXR inhibitor T-β-MCA, treatment of mice with a microbial BSH-resistant synthetic iFXR inhibitor, glycine-β-MCA, also reduced blood glucose and insulin levels (Jiang et al. 2015b). These effects were associated with reduced systemic ceramide levels and could be reversed by ceramide administration. Similar beneficial results were obtained by administering caffeic acid phenethyl ester (CAPE), which inhibits BSH and increases T-β-MCA levels. (Xie et al. 2017). Despite the fact that the translational relevance of these studies is limited, since humans do not produce muricholic acids, this work substantiates the claim that modulation of microbial species that control bile acid metabolism appears to be a viable approach for targeting host metabolism.

4.3 Indirect Pharmacological Approaches That Target iFXR

Another viable approach to modulate iFXR activity is through manipulation of intestinal bile acid transporters. In this scenario, *Asbt* deficiency or Abst inhibition will have an antagonizing effect, whereas *Ostα*/ *Ostβ* deficiency or blockade will result in activation of iFXR. Indeed, *Asbt*-deficient mice show increased fecal bile acid excretion, decreased ileal *Fgf15* expression, increased hepatic *Cyp7a1* expression, and a bile acid pool predominantly consisting of cholic acid. In contrast, *Ostα*-deficient mice show increased ileal *Fgf15* expression, suppressed hepatic *Cyp7a1* expression (reviewed in Dawson 2017), and decreased lipid absorption accompanied by modestly improved insulin sensitivity (Wheeler et al. 2014). Despite the fact that *Ostα*-deficient mice suffer from bile acid-induced hepatic injury in the postnatal period (Ferrebee et al. 2018), approaches to stimulate iFXR by pharmacological inhibition of OSTα/OSTβ have recently been investigated, supporting a role for this transporter as a drug target and its inhibitor clofazimine as a modulator of iFXR (van de Wiel et al. 2018).

Remarkably, inhibition of ASBT, which prevents iFXR activation, has also been shown to be beneficial for glycemic control (Chen et al. 2012; Wu et al. 2013; Rao et al. 2016), similar to bile acid sequestrants, which decrease iFXR signaling as a consequence of reduced bile acid reabsorption (Handelsman 2011). Pharmacological interventions such as bile acid-binding resins and ASBT or OSTα-β inhibitors increase fecal bile acid loss by preventing their reuptake by enterocytes or their subsequent release into portal blood. Obviously, potential adverse effects of an increased exposure of the colon to bile acids need to be taken into account during evaluation of these approaches. Increased fecal loss of bile acids will be compensated for by increased hepatic bile acid synthesis from cholesterol, thus inducing cholesterol synthesis and hepatic LDL receptor expression and accelerating LDL uptake from the blood compartment (Slijepcevic and van de Graaf 2017). ASBT inhibition was shown to reduce hepatic lipid accumulation in high-fat diet-fed

mice (Rao et al. 2016), although this beneficial effect could also result from fat malabsorption due to a decreased bile acid pool.

5 Perspective

Beyond its well-known functions in regulating bile acid homeostasis and ensuring cellular protection from bile acid toxicity, intestinal FXR is clearly also involved in the regulation of various aspects of energy metabolism, cellular proliferation, and intestinal barrier function, among others. These pleiotropic effects favor intestinal FXR as a potentially attractive target for a variety of diseases but, at the same time, also provide a basis for relevant adverse effects. In particular, the mitogenic risk of intestinal FXR modulation is of concern. More studies are needed to reconcile discordant findings surrounding intestinal FXR targeting in energy metabolism and to define the role of FXR in each relevant disease state. The translational value of murine studies must be verified for each pathway and disease under evaluation. The generation of mouse models with a "humanized" bile acid pool will constitute a valuable first step in clarifying some of these issues (Takahashi et al. 2016). Particularly, the consequences of iFXR modulation on intestinal carbohydrate metabolism require additional studies since, in our opinion, this aspect of iFXR biology has not sufficiently been addressed so far. Also, the potential consequences of posttranscriptional modulation of iFXR have not been sufficiently addressed to date. Importantly, as humans have highly varying inter-individual bile acid and microbiota profiles, "personalized medicine" approaches are likely to be relevant for effective intestinal FXR modulation. Notably, the only treatment with proven therapeutic benefits in humans so far, both with respect to hypercholesterolemia and glucose metabolism, is based on sequestration of bile acids, i.e., reduction of iFXR activation. Last but not least, the long-term effects of intestinal FXR agonism and/or antagonism need to be assessed, since for both beneficial effects have been reported in mouse studies, particularly with respect to the potential consequences of manipulating circadian rhythms of bile acid signaling.

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UDCA, NorUDCA, and TUDCA in Liver Diseases: A Review of Their Mechanisms of Action and Clinical Applications

Daniel Cabrera, Juan Pablo Arab, and Marco Arrese

Contents

Abstract

Bile acids (BAs) are key molecules in generating bile flow, which is an essential function of the liver. In the last decades, there have been great advances in the understanding of BA physiology, and new insights have emerged regarding the

D. Cabrera
Departamento de Gastroenterología, Escuela de Medicina, Pontificia Universidad Católica de Chile, Santiago, Chile

Departamento de Ciencias Químicas y Biológicas, Facultad de Salud, Universidad Bernardo O'Higgins, Santiago, Chile

J. P. Arab · M. Arrese (✉)
Departamento de Gastroenterología, Escuela de Medicina, Pontificia Universidad Católica de Chile, Santiago, Chile
e-mail: marrese@med.puc.cl

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role of BAs in determining cell damage and death in several liver diseases. This new knowledge has helped to better delineate the pathophysiology of cholestasis and the adaptive responses of hepatocytes to cholestatic liver injury as well as of the mechanisms of injury of biliary epithelia. In this context, therapeutic approaches for liver diseases using hydrophilic BA (i.e., ursodeoxycholic acid, tauroursodeoxycholic, and, more recently, norursodeoxycholic acid), have been revamped. In the present review, we summarize current experimental and clinical data regarding these BAs and its role in the treatment of certain liver diseases.

Keywords
Bile acids · Bile flow · Cell injury · Cholestasis · Inflammation · Liver diseases · Signaling

1 Introduction

Bile acids (BAs) are amphipathic species composed of four steroid rings forming a hydrocarbon lattice having hydrophobic and hydrophilic regions, containing hydroxyl groups, within their structure (Hofmann and Hagey 2008; Hofmann 2009; Russell 2009). The balance between hydrophobic and hydrophilic characters varies markedly among different BAs, which account for differences in their biological properties including their choleretic potency, solubilization properties (Carey 1984), and activation of bile acid receptors (Monte et al. 2009; Hofmann and Hagey 2014). The number of hydroxyl groups (the hydrophilicity of a given bile acid is greater if the number of hydroxyl groups is higher) and its orientation (i.e., α or β orientation at position 3, 6, 7, and 12 on the steroid backbone) are critical in determining hydrophobicity (Carey 1984). Thus, while hydrophobic BAs (i.e., lithocholic acid (LCA), deoxycholic acid (DCA), chenodeoxycholic acid [CDCA], and cholic acid [CA]) are potent detergents, hydrophilic BAs (i.e., ursodeoxycholic acid [UDCA], tauroursodeoxycholic acid [TUDCA]) are not (Hofmann and Small 1967). More importantly, they lack membrane-disrupting properties being nontoxic to the liver cell even in high concentrations (Paumgartner and Beuers 2004; Ashby et al. 2018). This is relevant for the therapeutic use of hydrophilic BA in the treatment of liver diseases although a myriad of additional mechanisms may be at play (Arab et al. 2017a; Beuers et al. 1998; Lazaridis et al. 2001; Beuers 2006) particularly in the case of new semisynthetic bile acid derivatives such as 24-norursodeoxycholic acid (NorUDCA), which seem to exercise hepatoprotective actions by novel mechanisms (Halilbasic et al. 2017). In this chapter, we summarize current data on the mechanisms of actions underlying the beneficial effects of selected hydrophilic BA (Fig. 1) in liver diseases as well as the information on the present and future clinical applications of these compounds.

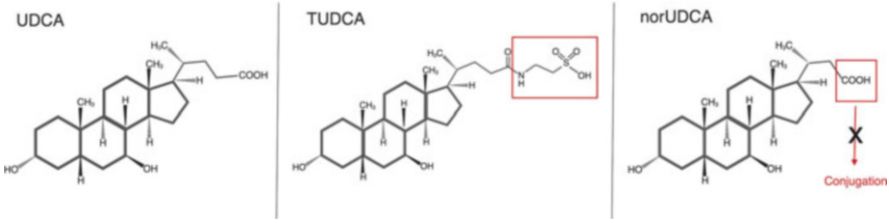

Fig. 1 Molecular structure of UDCA, TUDCA, and NorUDCA. Ursodeoxycholic (UDCA) is a hydrophilic dihydroxy (i.e., 3α,7β-dihydroxy-5β-cholan-24-oic acid) bile acid that represents 4% of bile acids in human bile. It likely originates in the colon by bacterial 7β epimerization of the primary bile acid chenodeoxycholic. The 17 carbon of UDCA may be amidated with glycine, which is the predominant pathway in humans, or with taurine, which is the predominant pathway in rodents. The taurine conjugate (TUDCA) has potent hepatoprotective actions. Norursodeoxycholic acid (NorUDCA) is a side chain-shortened (C23 instead of C25) synthetic bile acid with derivate from UDCA. NorUDCA is relatively resistant to amidation, which allow this compound to undergo cholehepatic shunting

2 Historical Remarks

The use of hydrophilic BA in the treatment of liver disease can be tracked back more than 1,000 years ago when ancient Chinese practitioners (Tang dynasty, 618–907 A.D.) discovered the therapeutic effects of bear bile in several conditions including chronic liver diseases (Hofmann and Hagey 2014; Marin et al. 2015; Li et al. 2016; Beuers et al. 2015a). Bear bile continues to be used until nowadays in some Asian countries where bile is obtained from farmed animals (Li et al. 2016), which poses some ethical issues considering the availability of alternative compounds (British Veterinary Association 2018).

In 1927, Shoda published his findings on a unique bile acid he found in bile of the Chinese black bear (Shoda 1927). This author named this bile acid as UDCA in reference to the Latin name of bear (ursus). Several years after, the structure of UDCA was better defined and the substance synthetized for its use in research (Lazaridis et al. 2001). Then in the 1950s, it was proposed that the beneficial effects of the bear bile were likely related to the high concentrations of the taurine-conjugated form of UDCA and TUDCA observed in that fluid (Li et al. 2016). Further research showed that UDCA and TUDCA were found to be potent choleretic agents when infused to rats (Hofmann and Hagey 2008; Makino and Tanaka 1998), and Japanese researches first investigated its use in chronic liver disease (Mijayi et al. 1976; Yamanaka et al. 1976). Later, in gallstone dissolution trials, the authors observed that, in contrast with chenodeoxycholic acid (CDCA), UDCA administration was not associated to liver toxicity (Ashby et al. 2018; Leuschner et al. 1985). For this reason, UDCA soon replaced CDCA for gallstone dissolution due to its similar efficacy and lack of hepatotoxicity (Paumgartner et al. 1994). With the advent of laparoscopic cholecystectomy, the use of BA for gallstone disease

decreased markedly, and interest in their biological properties and chemistry declined. It was in 1987 when the German hepatologist Ulrich Leuschner and coworkers (Leuschner and Kurtz 1987) reported beneficial effects of UDCA in patients with primary biliary cholangitis [PBC, a disease previously known as primary biliary cirrhosis (Beuers et al. 2015b)]. This, along with important advances made at the time in the understanding of the mechanisms at play in the generation and regulation of BA flux in the enterohepatic circulation (Blitzer and Boyer 1982; Coleman 1987), revamped the interest in the use of BA in the clinic. Subsequent studies by Poupon et al. (1987, 1991) and Lindor et al. (1994) paved the road to generate the evidence that support the routine use of UDCA as standard of care in PBC patients (Lindor et al. 2019; European Association for the Study of the Liver 2017). The use of UDCA in other cholestatic diseases although less evidence-based became common practice in the field of hepatology given the lack of toxicity of the drug.

After the journey that led UDCA to become an established drug in hepatology, studies with its taurine conjugate TUDCA have been conducted with similar results. However, in some basic and human studies, some differences were found (Setchell et al. 1996; Beuers et al. 1996), which led to new studies that derived in the potential use of TUDCA in neurodegenerative diseases (reviewed in Vang et al. 2014). More recently, considerable attention has been given to a parent compound of UDCA, norursodeoxycholic acid (NorUDCA), which is a side chain-shortened homologue of UDCA that is partially resistant to amidation, which theoretically enables its cholehepatic shunting (Halilbasic et al. 2017; Li and Lu 2018). This compound was developed after seminal work from Alan F. Hofmann in California (Schteingart and Hofmann 1988) whom confirmed the potent choleretic properties of NorUDCA (Hofmann et al. 2005) and predicted its clinical potential in cholestatic diseases. As described below, NorUDCA is a unique bile acid that has potential for treatment of several cholestatic and metabolic liver diseases. Studies with this bile acid as well as with other bile acid analogues have indeed represented an uptick in bile acid research, which opened new avenues for treatment of liver and biliary diseases.

3 Current Knowledge on the Mechanisms of Action of Hydrophilic Bile Acids in Liver and Biliary Diseases

An important body of information regarding the hepatoprotective and beneficial effects of hydrophilic BA on liver injury has been generated in the last three decades using different models of liver injury (Mariotti et al. 2018; Sharma et al. 2011). Also, research advances on the pathophysiology of cholestasis (Wagner and Trauner 2016; Jansen et al. 2017) and in the particular role of BA in determining cell injury (Perez and Briz 2009; Trauner et al. 2017) and death in both hepatocytes and cholangiocytes as well as in triggering an inflammatory response in the setting of cholestasis had led to a focused research on BA-mediated liver injury. This new knowledge had revamped bile acid-based therapeutic strategies for liver diseases (Arab et al. 2017a; Hegade et al. 2016). Although most of the available information

regarding the mechanisms underlying the hepatoprotective effects of hydrophilic BA has been tested in experimental models of cholestasis using either in vitro systems or whole animals (Mariotti et al. 2018, 2019), some of these hepatoprotective properties seem to operate also in other models of injury and eventually apply to metabolic diseases. In the following paragraphs, a brief summary of the importance of the hepatotoxicity of retained endogenous BA in cholestasis is provided as well as information on the particular features that explain the hepatoprotective properties of hydrophilic BA.

3.1 Core Concepts on Bile Acid Transport, Bile Acid-Induced Toxicity, and Hepatocellular Adaptive Responses in Cholestasis

The vectorial transport of BA by the hepatocytes involves several transport proteins and enzymes including the sinusoidal transporter sodium taurocholate cotransporting polypeptide (NTCP/SLC10A1), members of the anion transporting polypeptide (OATPs/SLCO) family, conjugation enzymes, and the ATP-dependent efflux pump BSEP (bile salt export pump [also known as ABCB11]) (Trauner and Boyer 2003; Halilbasic et al. 2013). These proteins allow a rapid transition of BA from blood to bile and maintain a low intracellular BA concentration (estimated in the micromolar range). This is crucial to maintain hepatocyte integrity as BAs are signaling and detergent molecules that at higher concentration ($\geq$50 μM or mM concentrations) may cause apoptosis, activate pro-inflammatory genes, and eventually induce cellular necrosis (Jansen et al. 2017; Li et al. 2017a; Woolbright and Jaeschke 2016). This inherent cytotoxicity of BA plays a role in liver damage in cholestatic conditions where bile secretion is impaired and BA accumulate inside hepatocytes and, in the case of cholangiopathies, leak into the surrounding tissue due to injury of bile ducts (Jansen et al. 2017). Of note, in the cholestatic setting, changes in the expression of hepatobiliary transporters occur that may represent a compensatory response aiming to limit the accumulation of potentially toxic biliary constituents (Arrese and Trauner 2003). These changes include downregulation of BA uptake, downregulation of BA synthesis, and upregulation of BA excretion through increased BSEP or transporters able to provide alternative excretory routes (Wagner et al. 2010; Arrese and Karpen 2010). These adaptive responses are mediated by the activation of several nuclear receptors such as farnesoid X receptor (FXR), pregnane X receptor (PXR), Constitutive Androstane Receptor (CAR), and the small heterodimer partner (SHP) as well as by entero-hormones such as Fibroblast growth factor 19 (FGF19), which is produced in the ileum and also in hepatocytes (in humans) (Halilbasic et al. 2013; Arrese and Karpen 2010). FXR is a major player and is a dedicated BA receptor that influences a myriad of pathways both in hepatocytes and in other resident cells such as Kupffer, endothelial, and hepatic stellate cells (Matsubara et al. 2013). In hepatocytes in particular, upon upregulation of SHP, FXR mediates a downregulation of NTCP and of cholesterol 7α-hydroxylase (CYP7A1), a key enzyme in BA synthesis. FXR also directly

upregulates BSEP, thus promoting BA excretion (Halilbasic et al. 2013). In humans, but not in mice, hepatic production of FGF-19 may also play a role in down-regulating CYP7A1 (Jansen et al. 2012). Finally, alternative excretory transport proteins located at the basolateral membrane of hepatocytes (i.e. the heteromeric transporter Organic solute transporter α-β [OST-α-β] and the ABC transporters MRP3, and MRP4) that are expressed at low levels in physiological conditions become upregulated during cholestasis (Halilbasic et al. 2013). Thus, if BA secretion is impaired, adaptive responses may limit BA accumulation inside hepatocytes, thus preventing hepatocellular damage. If these responses are insufficient, cell damage and death may occur either by apoptosis or necrosis (Woolbright and Jaeschke 2016). Of note, it has been shown that cholestatic hepatocytes can trigger hepatocyte-specific inflammatory response that involves increased expression of cytokines such as C-C Motif Chemokine Ligand 2 (CCL2), Chemokine (C-X-C motif) ligand 2 (CXCL2), and Interleukin 8 (IL-8) that in turn can contribute to neutrophil recruitment and augment local inflammation (Li et al. 2017a; Cai et al. 2017). This response is partially dependent on activation of toll-like receptor-9 presumably by BA-induced mitochondrial damage and the release of mitochondrial DNA (Cai et al. 2017). In addition to the local inflammation promoted by BA in other scenarios such as in cholangiopathies or bile duct diseases, mechanical obstruction leads to increased biliary pressure and the occurrence of biliary infarcts and the leak of BA and other biliary constituents into surrounding tissue that may activate proliferative reactions and hepatic fibrogenesis leading to disease progression and ultimately to cirrhosis (Jansen et al. 2017).

3.2 Bile Acids and Cholangiocytes in Cholestasis

Advances in the pathobiology of biliary epithelia have also been significant in the last two decades (Cheung et al. 2017; Han et al. 2013; Banales et al. 2019). Cholangiocytes, the epithelial cells lining the intra- and extrahepatic biliary tree, are heterogeneous polarized cells that contain a significant amount of transport proteins that allow the secretion of large amounts of bicarbonate (via the Cl^-/HCO_3^- exchanger (anion exchanger 2 [AE2])), water (through aquaporin-1 [AQP-1]), and chloride (through the low conductance cystic fibrosis transmembrane conductance regulator [CFTR]) that enrich canalicular bile and contribute to regulate biliary pH, which is important for activation of pancreatic enzymes and the absorption of lipophilic organic compounds. Cholangiocytes also express BA transporters (the apical sodium-dependent bile acid transporter [ASBT] is present in the apical membrane, and a truncated form of the same transporter [referred to as t-Asbt] is located at basolateral membrane of cholangiocytes) that allow for reabsorption of conjugated BA. It is important to note that cholangiocytes exhibit morphological, biochemical, and functional heterogeneity throughout the biliary system (i.e., from small to large bile ducts) with different cellular processes taking place at different locations of the biliary tree (Banales et al. 2019). Also, passive absorption of protonated unconjugated BA can occur. The reuptake of BA in cholangiocytes

followed by re-secretion into the blood of peribiliary plexuses is referred as the "cholehepatic shunt pathway," which leads to BA return to hepatocytes for re-secretion into bile augmenting its choleretic action. Finally, some in vitro and in vivo evidence suggest that biliary BA concentration and composition may eventually regulate some cholangiocyte functions by activating differing signaling pathways and (i.e., calcium protein kinase C [PKC], phosphoinositide 3-kinase [PI3K], mitogen-activated protein [MAP] kinase, and extracellular signal-regulated protein kinase [ERK], among others), thus inducing changes in cholangiocyte secretion, proliferation, and survival. It has been also shown that cholangiocyte proliferation is critically dependent of the BA receptor TGR5, which is located in the cholangiocyte cilia.

Cholangiocyte injury is a key phenomenon in certain cholestatic diseases, and therefore aspects related to cholangiocyte responses to injury are also of importance to the understanding of cholestasis pathophysiology and treatment (Banales et al. 2019; Sato et al. 2018). When injured, cholangiocytes respond acquiring a neuroendocrine phenotype and, in response to a myriad of stimuli, proliferate leading to bile duct hyperplasia, which is a common histological hallmark of cholestatic diseases (Cheung et al. 2017). Injury of biliary cells can be immune-mediated, toxically induced, or related to mechanical factors (i.e., biliary obstruction). In all these settings, direct cytotoxicity of BA could play a role as increased luminal BA can damage cholangiocyte membrane, induce autophagy, and promote cellular senescence, which is associated to secretion of pro-inflammatory and pro-fibrotic signals (Cheung et al. 2017; Xia et al. 2006). Bicarbonate secretion and the existence of an intact cholangiocyte glycocalyx have been hypothesized to form a "bicarbonate umbrella" that prevents protonation of biliary BA and cellular damage by bile acid monomers (Beuers et al. 2015a; Hohenester et al. 2012).

3.3 Mechanisms Underlying the Hepatoprotective Properties of Hydrophilic Bile Acids

Based on the information summarized above, strategies that have been exploited (Beuers et al. 2015a; Wagner and Trauner 2016; Trauner et al. 2017) therapeutically for cholestatic diseases include the following: (a) to limit BA injury through modulation of BA pool hydrophobicity or reducing bile acid pool size by interfering with intestinal bile acid absorption, (b) to induce choleresis to deload hepatocytes from BA and to limit cholangiocyte damage, and (c) to modulate inflammation. Hydrophilic BA can exercise some of these functions, which explain their usefulness in liver diseases (Figs. 2 and 3). Their effects on the hepatobiliary system are summarized below.

3.3.1 UDCA and TUDCA

UDCA (3α,7β-dihydroxy-5β-cholanoic acid) is normally present in human bile, amounting to 1–3% of biliary BA (Marin et al. 2015). In physiological conditions most of the UDCA is conjugated with glycine, which is the preferred amidation

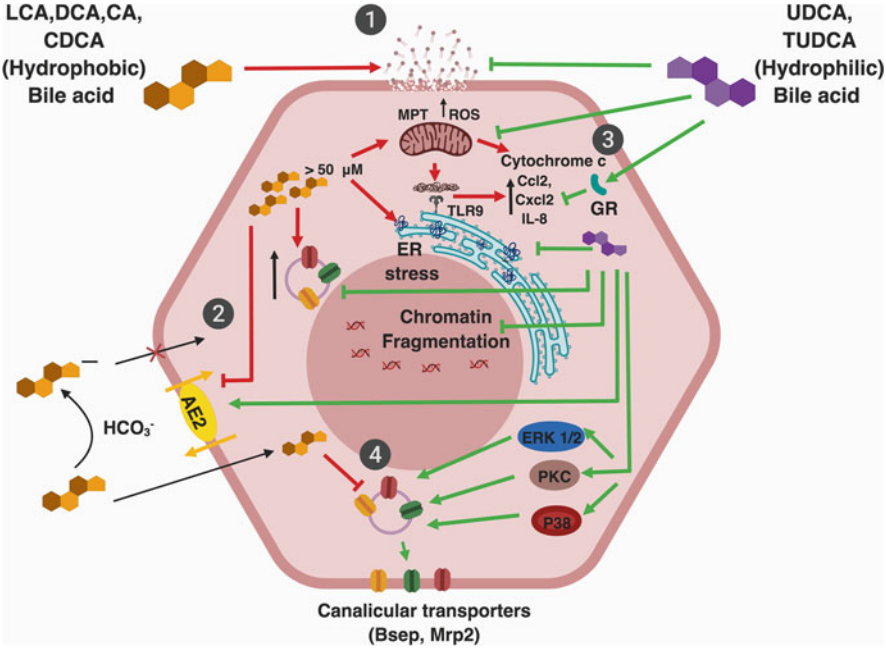

Fig. 2 Overview of the main hepatoprotective mechanisms of action of UDCA and TUDCA. In cholestasis, hydrophobic bile acids induce many cellular changes that can be counteracted by hydrophilic bile acids such as UDCA and TUDCA. (1) Hydrophobic bile acids are strong detergents that can cause membrane disruption by lipid solubilization, while hydrophilic bile acids like UDCA can bind to the apolar domain of cell membranes, stabilizing its molecular structure. (2) Bicarbonate secretion by hepatocytes and cholangiocytes has protective action against the detergent effects of hydrophobic bile acids. Treatment with hydrophilic bile acids induces bicarbonate secretion by several mechanisms including increasing of the anion exchanger 2 [AE2] expression. AE2 exchanges chloride by bicarbonate in both hepatocytes and cholangiocytes. (3) Hydrophilic bile acids can also inhibit apoptotic signaling pathways at the level of mitochondria or indirectly through anti-inflammatory effects by binding the glucocorticoid receptor (GR) and counteracting the pro-inflammatory effects of bile acids, which are mediated by toll-like receptor 9 (TLR9). (4) Cholestasis induces endocytic internalization of canalicular transporters, like the bile salt export pump (BSEP) and the multidrug resistance-associated protein 2 (MRP2). Treatment with hydrophilic bile acids increases the translocation of transporters such as BSEP and MRP2 into the canalicular membrane

pathway in humans (Hofmann 2009). Oral administration of UDCA is able to enrich the biliary bile acid pool with this hydrophilic bile acid up to 40% of biliary BAs (Rost et al. 2004; Dilger et al. 2012), which is thought to decrease BA pool hydrophobicity and therefore reduce its hepatotoxic effects if hepatocyte BA retention occurs. This was thought to be central to the effects of UDCA in cholestatic diseases given the role of hepatic retention of hydrophobic bile acids as a major cause of liver damage, by inducing membrane damage, necrosis, and apoptosis, in this setting (Wagner and Trauner 2016; Arrese and Trauner 2003). However, one

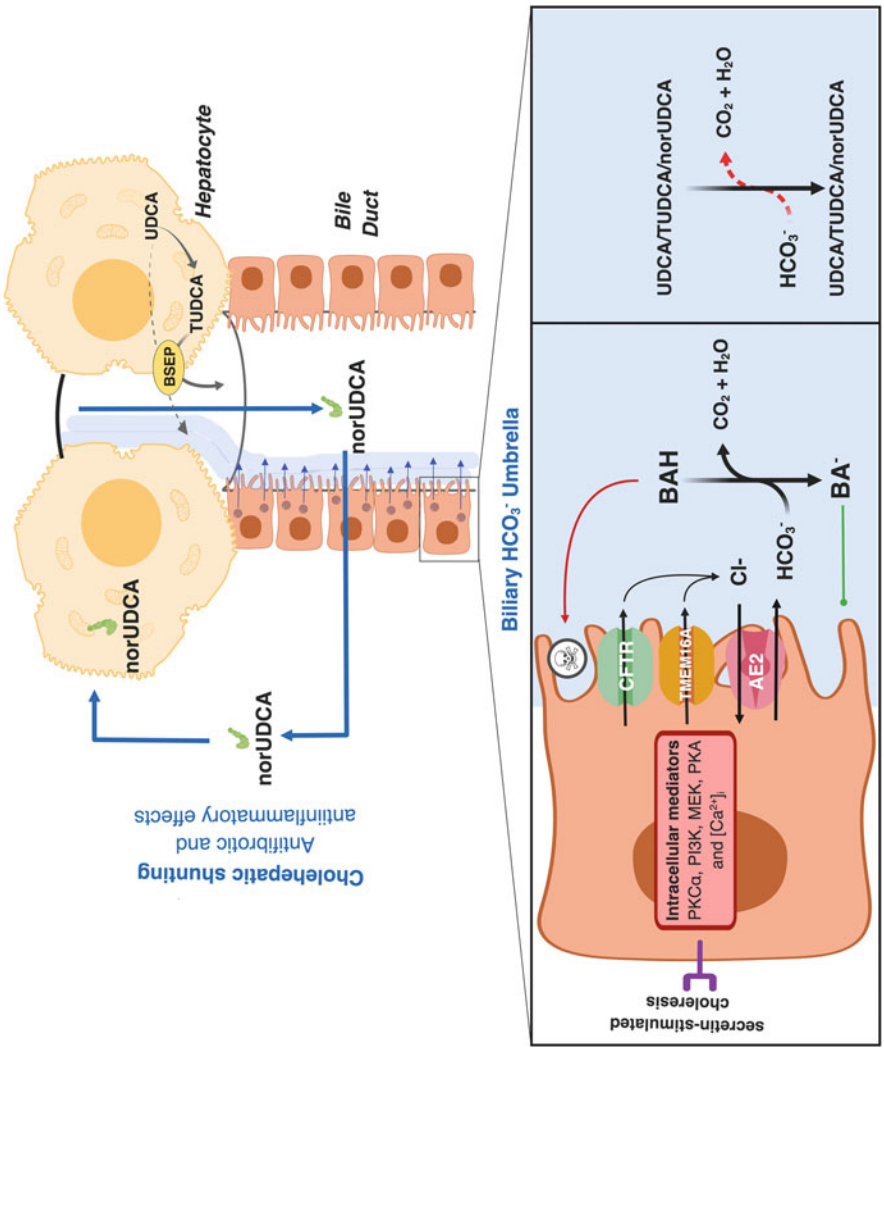

Fig. 3 The biliary HCO_3^- umbrella hypothesis and the hepatoprotective actions of TUDCA, UDCA, and NorUDCA. Hydrophobic bile acids are toxic to cholangiocytes, inducing senescence, endoplasmic reticulum stress, autophagy, and cell death. These effects are counteracted by bicarbonate secretion via the

Fig. 3 (continued) Cl^-/HCO_3^- anion exchanger 2 [AE2] in hepatocytes and cholangiocytes and likely also by transmembrane member 16A (TMEM16A) in cholangiocytes. UDCA, TUDCA, and NorUDCA have been observed to have protective effects in the liver by preventing bicarbonate depletion on the apical side of cholangiocytes, thus exercising cytoprotective effects. Particularly, NorUDCA, due to its resistance to amidation, can undergo cholehepatic shunting and potently promote bicarbonate secretion into the bile duct. Although most of the UDCA is secreted as a glycine conjugate in humans, taurine conjugation predominates in rodents. Secretion of TUDCA is mediated by the canalicular bile salt export pump (BSEP)

report (Beuers et al. 1992) showing that hydrophobic BA pool sizes remained stable during short treatment with UDCA suggests that this would not be the major mechanism of action of the drug. Rather, UDCA-induced signaling changes in hepatocytes that modulate relevant pathways for hepatobiliary secretion, cellular stress, and apoptosis would underlie the hepatoprotective effects of UDCA (Beuers et al. 2015a). Of note, UDCA seems to exert its beneficial effects mainly at the level of hepatocytes and cholangiocytes. Additional effects may be related to some actions at the intestinal level since amidated forms of UDCA inhibit gut absorption of endogenous bile acids (Lanzini et al. 2003). The multiple mechanisms of action described for UDCA (reviewed in depth in refs. Beuers 2006; Beuers et al. 2015a) are delineated below.

Actions of UDCA/TUDCA in Hepatocytes

Most of the data regarding the effects of UDCA in the liver has been generated using the nonconjugated form as well as its taurine conjugate TUDCA with few differences between them. At the level of hepatocytes, UDCA has been shown to stimulate bile flow as well as the secretion of organic anions (Beuers 2006). Early studies with continuous intravenous infusion of TUDCA in whole animals (Kitani and Kanai 1981, 1982) and perfused rat livers (Beuers et al. 1993a) showed that the UDCA induced higher flow rate and higher total bile salt secretion than taurocholate. Further studies suggested that UDCA-induced choleresis is attained through post-transcriptional actions that lead to increased insertion of transporters such as BSEP and MRP2 into the canalicular membrane (Kurz et al. 2001; Beuers et al. 2001) and stimulation of hepatic bicarbonate secretion, which stimulates the secretion of an alkaline bile (Takikawa et al. 1992). Some of these effects may be related to a potent stimulation of intracellular Ca2+ signaling (Beuers et al. 1993a, b) and other pathways such as that of protein kinase C (cPKCa) (Beuers et al. 1996; Stravitz et al. 1996), mitogen-activated protein kinases (MAPK, Erk1/2, p38MAPK), and alpha-5-beta-1 integrins in hepatocytes (Haussinger and Kordes 2017). Importantly, the effect of UDCA on hepatic transport protein expression in vivo is rather modest stressing the relevance of posttranscriptional effects of UDCA as responsible of some of its beneficial effects in the liver (Fickert et al. 2001). Of note, it has been also shown that UDCA also prevents the endocytic internalization of canalicular transporters, a common feature in cholestasis (Roma et al. 2011). Finally, it must be kept in mind that all the abovementioned effects have been shown only in experimental models and is uncertain to which extent they operate in humans (Beuers et al. 2015a).

Other important mechanism by which UDCA is hepatoprotective is related to its antiapoptotic activity. Cellular toxicity of BA is in part related to apoptosis induction, and UDCA and TUDCA have been shown to inhibit classic pathways of apoptosis (Amaral et al. 2009; Azzaroli et al. 2002; Benz et al. 1998; Rodrigues and Steer 2001). Using primary rat hepatocytes and HUH-7 hepatoma cell lines, Rodrigues et al. showed that, in contrast to DCA, UDCA is innocuous in terms of apoptosis induction (Rodrigues et al. 1998a). When the two bile acids were combined in the diet, UDCA completely inhibited cell death by apoptosis associated with

the hydrophobic bile acid alone. UDCA can abolish typical morphological changes of apoptotic nuclei like nuclear fragmentation of condensed chromatin. Also, UDCA has been shown to inhibit apoptosis induction driven by ethanol, TGF-β1, FAS ligands, and okadaic acid (a robust apoptotic stimulus) suggesting a ubiquitous antiapoptotic effect of UDCA (Amaral et al. 2009). Additional reports have shown that UDCA markedly reduces mitochondrial release of cytochrome c into the cytoplasm, in liver cells treated with hydrophobic BA by inhibition of both channel-forming activity and depolarization of the mitochondrial membrane (Rodrigues et al. 1998a, 1999). These findings support the concept that UDCA can modulate the apoptotic threshold by its protective role over mitochondrial membrane perturbation (Rodrigues et al. 1998a, b). In addition, activation of survival pathways such as p38, ERK, MAPK, and PI3K pathways has been also demonstrated in vitro (Schoemaker et al. 2004). Finally, it has been described that UDCA and TUDCA also attenuate endoplasmic reticulum stress by acting as cellular chaperones (Ozcan et al. 2006), which may also account for the antiapoptotic effects of these BA. Of note, the antiapoptotic properties of UDCA and its taurine conjugate TUDCA have been demonstrated also in other cell types particularly in neurons (Abdelkader et al. 2016). These effects are currently being explored with therapeutic purposes in some neurodegenerative diseases such as Parkinson's diseases and lateral amyotrophic sclerosis (Vang et al. 2014; Abdelkader et al. 2016; Castro-Caldas et al. 2012; Elia et al. 2016).

In addition to its effects on hepatobiliary transport capacity and to its antiapoptotic properties, existing data also support some action on oxidative injury as well as membrane stabilization and anti-inflammatory effects. Mitsuyoshi et al. assessed the effects of UDCA on oxidative injury and antioxidative systems in cultured rat hepatocytes. They found that UDCA significantly prevented cellular damage after hydrogen peroxide or cadmium challenge (Mitsuyoshi et al. 1999). UDCA also increased the amounts of glutathione (GSH) and thiol-containing proteins as well as the mRNA levels of γ-glutamylcysteine synthetase suggesting hepatoprotective effects against oxidative injury. With regard to membrane stabilization, experimental in vitro data from Guldutuna et al. proposed that UDCA can bind to the apolar domain of cell membranes stabilizing its structure and avoiding the lipid solubilization induced by hydrophobic BA such as CDCA (Guldutuna et al. 1993). More recent evidence suggest that UDCA prevents damaging effects of hydrophobic BA only in the presence of membrane cholesterol (Zhou et al. 2009). Finally, UDCA has been described as a ligand of glucocorticoid receptor, which could be related to an anti-inflammatory effect (Tanaka and Makino 1992; Miura et al. 2001), and has been postulated to have some immunomodulatory effects (Yoshikawa et al. 1992). The relevance of the UDCA actions described above remains unclear in the human setting.

Actions of UDCA/TUDCA in Cholangiocytes

As mentioned earlier, cholangiocyte injury is a key phenomenon in certain cholestatic diseases contributing to local inflammation and fibrosis development (Sato et al. 2019; Fabris et al. 2017). Among the phenomena involved in cholangiocyte damage in

cholangiopathies are direct effects of hydrophobic bile acids on cell membrane, activation of autophagy, and induction of senescence as well as induction of endoplasmic reticulum stress and immune-mediated injury (Banales et al. 2019; Sasaki and Nakanuma 2017). Clinical and experimental data indicates that UDCA may act modulating these phenomena. Current concepts are summarized below.

Several authors have pointed to the importance of bicarbonate secretion for the protection of cholangiocytes against the damaging effect of protonated BA present in bile. Conceptually, bicarbonate secretion increases biliary pH and determines a shift of BA toward ionized forms, thus decreasing their ability to diffuse and reducing their cytotoxic effects (Banales et al. 2019). This concept has been stated as the "biliary bicarbonate umbrella hypothesis" (Hohenester et al. 2012; van Niekerk et al. 2018), which is thought to be defective in cholangiopathies such as PBC (Rodrigues et al. 2018). Biliary bicarbonate secretion is carried out by AE2 (SLC4A2), which is expressed in the apical membrane of cholangiocytes and depends on active chloride secretion by these cells. Of note, both messenger RNA and protein levels of AE2 as well as biliary bicarbonate secretion are reduced in PBC, a prototypic cholestatic disease (Prieto et al. 1993, 1999). UDCA treatment determines increased fluid secretion from cholangiocytes as well as increases biliary bicarbonate secretion via activation of [AE2] and also transmembrane member 16A (TMEM16A) (Fiorotto et al. 2007; Li et al. 2018). Also, UDCA restores cholestasis-associated reduced AE2 mRNA and protein expression, which is thought to be an important mechanism of action of UDCA in cholangiopathies. Importantly, recent evidence suggests that dysregulated autophagy and cholangiocyte senescence in PBC may also be related to a defective biliary bicarbonate umbrella since AE2 knockdown evokes these phenomena in biliary cells (Sasaki et al. 2018). Additional effects of UDCA in cholangiocytes include restoration of secretin-stimulated choleresis via multiple mediators (i.e., AE2, PKCα, PI3K, MEK, PKA, and intracellular Ca2+) (Uriz et al. 2011; Jones et al. 2015). More recently, two new players have been added to the list of potential mediators of the beneficial effects of UDCA in cholangiocytes. On one hand, a recent study showed that the bile acid sensitive ion channel (BASIC), which is highly expressed in cholangiocytes, is strongly activated by UDCA (Wiemuth et al. 2013). On the other hand, Li et al. recently show that both UDCA and TUDCA stimulate Cl_2 secretion through activation of TMEM16A, which is thought to be, instead of CFTR, the dominant Cl_2 channel regulating anion efflux in biliary epithelia (Li et al. 2018). While further studies are needed to better determine the role and cellular activities of these channels, the abovementioned studies suggest that the therapeutic effects provided by UDCA might be related to modulation of their channel activities.

Effects of UDCA on Gut Microbiome

Information on the role of microbiome in chronic liver disease has surged in recent years (Wahlstrom 2019), but the existence of many confounders in available studies makes clear conclusions difficult to reach. Indeed, there is a close and bidirectional interplay between BA metabolism and the gut microbiota, and cholestasis may alter intestinal bacterial populations. Recent studies have explored the effects of UDCA

on gut microbiome composition in healthy subjects and also in individuals with liver dysfunction (Kim et al. 2018; Pearson et al. 2019). Interestingly, UDCA influenced bacterial populations inducing marked decrease in abundance of *Bifidobacterium*, *Lactobacillus*, and *Lactobacillaceae* (Kim et al. 2018). It remains to be determined if these effects have any relevance for the therapeutic action of UDCA. One interesting recent study showed that the absence of the intestinal microbiota results in exacerbation of liver injury in a murine model of primary sclerosing cholangitis (PSC), the *mdr2−/−* mice (Tabibian et al. 2016). This genetically engineered mouse is deficient of the canalicular transporter of phospholipid and has very low levels of biliary phosphatidylcholine, which results in biliary injury. The biliary alterations of this experimental model are similar to that observed in PSC (Mariotti et al. 2019). In the study by Tabibian et al. (2016), germ-free *mdr2−/−* mice exhibited significantly worse liver chemistry and histological lesions than conventionally housed mice underscoring the importance of commensal microbiota in protecting against biliary damage.

Few studies have analyzed the gut microbiome in cholestatic diseases (Quigley 2016; Li et al. 2017b). Of note, a significant reduction of within-individual microbial diversity has been found in PBC (Tang et al. 2018a), which is partially relieved by UDCA administration. Similarly, reduced diversity and significant shifts in the microbiome composition have been found in stool samples from PSC patients (Kummen et al. 2017), but it is unclear if they are primary or secondary to the bile secretory failure present in cholestatic disorders.

3.3.2 NorUDCA

As mentioned earlier, 24-norursodeoxycholic (NorUDCA) is a non-amidated, side chain-shortened C23 derivative of UDCA that in virtue of its relative resistance to amidation undergoes biliohepatic shunting being a potent choleretic compound due to the amplification of its effect on bicarbonate secretion by cholangiocytes (Halilbasic et al. 2017; Trauner et al. 2015; Yoon et al. 1986). NorUDCA has been shown to have profound beneficial effects in experimental models of biliary injury particularly in the *mdr2−/−* mice. In this mouse model, NorUDCA exercise marked beneficial effects by reducing injury and biliary fibrosis (Halilbasic et al. 2009; Fickert et al. 2006). It is thought that the lack of biliary phospholipid facilitates cholangiocyte injury by hydrophobic BA and that this phenomenon is counteracted by a bicarbonate-rich bile induced by NorUDCA administration (Halilbasic et al. 2017). Of note, biliary bicarbonate enrichment induced by NorUDCA is much stronger than its parent compound UDCA (Trauner et al. 2017). In addition to the induction of bicarbonate-rich bile, NorUDCA seems to have some relevant immunological actions since it has been shown that it is able to affect antigen presentation and inhibit T-lymphocyte proliferation in a mouse model of schistosomiasis (Sombetzki et al. 2015). Also, antifibrotic effects have been described in the thioacetamide-induced liver fibrosis rat model (Buko et al. 2014) although the underlying mechanisms are unclear.

Table 1 Current therapeutic uses of hydrophilic bile acids in liver diseases

Efficacy established	Efficacy likely	Efficacy uncertain
UDCA/TUDCA for primary biliary cholangitis UDCA for cholesterol gallstone dissolution UDCA for cholestasis of pregnancy	UDCA for prevention of gallstones after bariatric surgery UDCA for low phospholipid-associated cholelithiasis UDCA for progressive familial intrahepatic cholestasis type 3 UDCA to prevent liver disease in cystic fibrosis UDCA to prevent hepatic injury after stem cell transplantation NorUDCA for primary sclerosing cholangitis	UDCA for drug-induced cholestatic liver injury UDCA to prevent total parenteral nutrition-induced cholestasis UDCA for primary sclerosing cholangitis UDCA and NorUDCA for NAFLD/NASH

UDCA ursodeoxycholic acid, *TUDCA* tauroursodeoxycholic acid, *NorUDCA* norursodeoxycholic acid, *NAFLD/NASH* nonalcoholic fatty liver disease/nonalcoholic steatohepatitis

4 Clinical Applications of Hydrophilic Bile Acids in Liver and Biliary Diseases: Current Status and Perspectives

Hydrophilic BA has been studied in different liver diseases. While ample evidence is available for UDCA, the amount of studies carried out with TUDCA and NorUDCA are less abundant. As shown in Table 1, there are settings in which these drugs can be considered clinically useful and other that await further confirmation. A summary of current data is provided below.

4.1 Efficacy Established

Several clinical uses of UDCA or its taurine conjugate have been proven in prospective clinical trials. This prerequisite is met for PBC, cholesterol gallstone disease, and intrahepatic cholestasis of pregnancy.

UDCA/TUDCA for PBC After the seminal reports of the effects of UDCA on liver test in patients with PBC (Leuschner and Kurtz 1987), most of the reports published confirmed the positive effects on liver markers of cholestasis particularly on serum alkaline phosphatase levels, which is currently considered a surrogate marker of outcomes in patients with PBC (Lammers et al. 2014). However, the utility of UDCA in PBC remained a matter of debate due to the lack of evidence of efficacy on hard end points (i.e., survival or liver transplantation (LT)-free survival). To prove benefit on these outcomes remained difficult due to the long natural history of the disease and the lack of power of studies that include small number of patients as well as patients with different disease stages. The latter is relevant as patients with

earlier histologic stage may respond better to UDCA than patients with more advanced disease stage (Ali et al. 2017).

Although analysis of pooled cohorts of treated patients comparing outcomes with the predicted survival by mathematical models suggested that UDCA prolonged LT-free survival (Lindor et al. 1994; Poupon et al. 1997), several meta-analysis, including a recent report of the Cochrane hepatobiliary group, concluded that there is no demonstrated benefit of UDCA on LT-free survival and/or mortality (Saffioti et al. 2017; Goulis et al. 1999). In spite of that, UDCA at a dose of 13–15 mg/kg/day is the recommended first-line treatment of PBC in current guidelines (Lindor et al. 2019; European Association for the Study of the Liver 2017) based on indirect evidence that the drug slows disease progression and reduces the need of LT (Lindor et al. 2019). Response is monitored using serum alkaline phosphatase levels, and those patients that reduce this parameter significantly (greater than 25% of the basal level) are considered responders with a complete response defined as normalization of serum alkaline phosphatase levels (Pares et al. 2000). Several other criteria for response have been published and validated (Ali et al. 2017). A more recent study from the Global PBC Study Group database including data from 3,902 patients confirmed that UDCA confers a survival benefit for PBC patients even for patients with incomplete response (Harms et al. 2019). These findings provide further support to the use of UDCA as standard medical therapy for PBC.

Most of the clinical studies carried out with UDCA have used the unconjugated form of the drug, which undergoes extensive conjugation primarily with glycine, before being excreted into bile (Crosignani et al. 1996). The use of the taurine-conjugated form of UDCA has been less common although, theoretically, it could have some potential advantages related to a greater hydrophilicity and reduced biotransformation to more hydrophobic metabolites (Setchell et al. 1996; Invernizzi et al. 1999). A recent study from China shows that TUDCA is equally safe and efficacious as UDCA with regard to its effects on serum levels of alkaline phosphatase in patients with PBC (Ma et al. 2016).

UDCA and TUDCA for Cholesterol Gallstone Disease Before the introduction of laparoscopic cholecystectomy, several nonsurgical treatments of gallstone disease were attempted. In the 1970s, it was demonstrated that BA could promote the dissolution of gallstones, and oral dissolution therapy using both CDCA and UDCA was studied in prospective clinical trials (Bell et al. 1972; Portincasa et al. 2012; Danzinger et al. 1972). UDCA became the drug of choice for this purpose since several studies demonstrated higher efficacy than CDCA in decreasing biliary cholesterol saturation as well as fewer side effects such as diarrhea or elevations of serum aminotransferases (Stiehl et al. 1978; Mok et al. 1974). At present time less than 10% of total patients are considered for gallstone dissolution therapy with UDCA (Portincasa et al. 2012) that can be suggested for symptomatic gallstone patients who are not eligible for surgery and have small (<5 mm in size), radiolucent stones in a functioning gallbladder with a patent cystic duct (Paumgartner et al. 1994). TUDCA is thought to be equally effective than UDCA for gallstone dissolution (Portincasa et al. 2012).

UDCA for Cholestasis of Pregnancy (ICP) Being a pregnancy-specific disorders, ICP occurs mainly in the third trimester of pregnancy and is characterized by pruritus and elevated bile acid levels with few cases developing jaundice (Arrese and Reyes 2006; Wood et al. 2018). The disease usually improves spontaneously after delivery (Wood et al. 2018). ICP is regarded as a benign disease with no meaningful consequences to the mother but associated to an increased perinatal risk with increased rates of fetal morbidity and mortality. The pathogenesis of the disease is unknown but likely involves a genetic hypersensitivity to estrogen or estrogen metabolites. Mutations or polymorphisms of some hepatobiliary transport proteins may contribute to disease pathogenesis or severity (Arrese et al. 2008). In addition to an adequate obstetric management to prevent fetal distress, UDCA is recommended to treat ICP. This is based on several prospective studies (Bacq et al. 2012; Palma et al. 1997) that showed beneficial effects on liver function test and resolution or improvement of pruritus in a significant proportion of patients (Bacq et al. 2012, 2017). Although the benefit of UDCA for reducing stillbirth in ICP remains unproven, its use as first-line therapy in ICP is recommended in current guidelines (Bicocca et al. 2018).

4.2 Efficacy Likely

Available studies suggest that the use of hydrophilic BA is likely effective in several other than the abovementioned diseases (Table 1). This is based on large clinical series and early clinical phase trials or inference from the observed effects of UDCA in other diseases. Evidence supporting these clinical uses are summarized below.

UDCA for Prevention of Gallstones After Rapid Weight Loss or Bariatric Surgery Based on the proven efficacy of UDCA in gallstone dissolution, its use in the prevention of gallstone formation in several clinical settings where bile become transiently lithogenic has been advocated. Among these conditions the effect of UDCA in prevention of gallstone disease after rapid weight loss and after bariatric surgery has been studied in formal clinical trials. Rapid weight loss (>1.5 kg/week) induces a supersaturated bile and determines gallstone formation in up to one third of bariatric surgery patients (Guzman et al. 2019). Updated meta-analysis suggests that UDCA administration significantly reduces gallstone formation in the setting of rapid weight loss induced either by very-low-calorie diets (Stokes et al. 2014) or bariatric surgery (Magouliotis et al. 2017) being a well-tolerated and safe medication. Thus, although the quality of evidence is moderate, administration of 500–600 mg of UDCA may be recommended during periods of rapid weight loss until body weight has stabilized. In the case of bariatric surgery, a period of 6 months after the surgical procedure is suggested (Magouliotis et al. 2017; European Association for the Study of the Liver 2016).

UDCA for Low Phospholipid-Associated Cholelithiasis (LPAC) LPAC is a rare condition characterized by low biliary phospholipid concentration, which

determines the occurrence of symptomatic and recurring cholelithiasis. This occurs usually before the age of 40 years with frequent concomitancy of intrahepatic bile duct and gallbladder cholesterol stones. The underlying causes are mutations in the ABCB4 gene that encodes the hepatocanalicular phospholipid transporter. LPAC patients may benefit from prophylactic UDCA therapy (15 mg/kg body weight per day) that seems to prevent the occurrence and recurrence of stones (European Association for the Study of the Liver 2016; Poupon 2012).

UDCA for Progressive Familial Intrahepatic Cholestasis Type 3 (PFIC3) PFIC3 is a genetic cholestatic disease seen in early life, which is also related to a defective expression or function of the hepatocanalicular phospholipid transporter due to mutations of the ABCB4 gene. As in LPAC, PFIC3 patients develop severe liver and cholangiocyte injury due to BA-mediated damage. Up to one third of patients may exhibit biochemical response to UDCA (Baker et al. 2019), but its use has not been proven in large clinical trials.

UDCA to Prevent Liver Disease in Cystic Fibrosis Patients with cystic fibrosis have a defective function of the cholangiocytes' low conductance chloride channel, CFTR. This leads to a reduced biliary bicarbonate and a "thick" bile, which in turn determine the formation of biliary plugs and the occurrence of biliary injury due to a defective biliary bicarbonate "umbrella." These phenomena trigger biliary obstruction and inflammation potentially resulting in biliary cirrhosis and portal hypertension (Sakiani et al. 2019; Assis and Debray 2017). However, only few patients develop symptomatic hepatobiliary disease although many CF patients have abnormal liver tests. If liver disease is present, the use of UDCA is recommended although its efficacy remains unproven due to the lack of high-quality studies. A recent Cochrane review performed concluded that currently available data to support the use of UDCA is limited (Cheng et al. 2017). Some studies suggest that if used early (i.e., before cirrhosis is established), UDCA could prevent or even alleviate liver damage in cystic fibrosis patients as estimated by a decrease in liver stiffness as measured by transient elastography (van der Feen et al. 2016). Given the lack of alternative therapies and safety of UDCA, most experts recommend its use in cystic fibrosis although more studies are needed to confirm its efficacy in preventing liver disease in patients with this disease (Sakiani et al. 2019).

UDCA to Prevent Hepatic Injury after Stem Cell Transplantation (HSCT) Hematopoietic stem cell transplantation is routinely used for management of many hematological disorders. A frequent complication of this therapy is acute graft-versus-host disease (GVHD), an immune-mediated disorder resulting in recipient tissue damage by immune cells from the donor. One prospective randomized study evaluated the use of UDCA administration for prevention of hepatic complications after HSCT. This study found that UDCA significantly reduced the proportion of patients developing hyperbilirubinemia and that UDCA-treated patients exhibited a reduced incidence of severe acute GVHD (Ruutu et al. 2014). However, no other

studies have been published regarding the use of UDCA in hepatic GVHD, and its efficacy in this setting remains to be proved.

NorUDCA for Primary Sclerosing Cholangitis Due to its novel mechanisms of action and the wealth of experimental evidence in preclinical models, the use of NorUDCA as a useful therapeutic agent in several liver disease holds promise (Halilbasic et al. 2017; Trauner et al. 2015). To date only the phase II clinical trial assessing the effects of NorUDCA in PSC patients has been published (Fickert et al. 2017). In this study, 161 PSC patients were randomized for a 12-week treatment followed by a 4-week follow-up. NorUDCA reduced serum levels of alkaline phosphatase in a dose-dependent manner in up to 26% with few serious adverse events. Moreover, NorUDCA significantly reduced serum levels of amino-transferases and gamma-glutamyl transferase. Although promising the real efficacy of NorUDCA in PSC remains to be proved in larger trials and with a more accurate patient stratification (Chazouilleres 2017). Currently, the use of NorUDCA in PSC patients is being evaluated in a phase III clinical study (ClinicalTrials.gov number NCT01755507).

4.3 Efficacy Uncertain

Due to its lack of favorable safety profile, UDCA and other hydrophilic BA have been used in a myriad of other liver diseases (Table 1) without proof of efficacy. Thus, although UDCA may be used, the usefulness of UDCA in the setting of drug-induced cholestatic liver injury (Sundaram and Bjornsson 2017) or parenteral nutrition-induced cholestasis (San Luis and Btaiche 2007), its use is debatable due to the lack of evidence. In the case of UDCA use in PSC patients, controlled trials have shown no efficacy (Lindor et al. 2015) although the drug might be useful in some subsets of patients at dose of 17–22 mg/kg/day (Tabibian and Lindor 2014). Higher doses (25–30 mg/kg/day) may be harmful (Sedki and Levy 2018).

UDCA and NorUDCA have been also proposed as potential treatment of nonalcoholic fatty liver disease (NAFLD) currently the more common liver disease worldwide (Younossi et al. 2018; Arab et al. 2017b). NAFLD and its progressive form nonalcoholic steatohepatitis (NASH) can led to advanced liver fibrosis in up to a quarter of patients (Arab et al. 2017b). Although robust evidence of beneficial effects of these hydrophilic BA in preclinical models of NAFLD/NASH has been published (Steinacher et al. 2017), UDCA was found to be ineffective in a large clinical trial and therefore is not recommended as treatment of NAFLD in current guidelines (Chalasani et al. 2018). In the case of NorUDCA, a yet unpublished recent phase II study showed beneficial effects in patients with NAFLD and elevated liver enzymes (Traussnigg et al. 2017). A larger trial is being conducted, and its results will eventually support the use of NorUDCA in NAFLD/NASH.

Finally, some studies in preclinical models have suggested that hydrophilic BA might influence cyst formation in the liver (Munoz-Garrido et al. 2015) as well as promote degradation of α1-antitrypsin mutant Z protein (Tang et al. 2018b) opening

the possibility of using UDCA in polycystic liver disease and alpha-1 antitrypsin deficiency, two rare liver diseases. Unfortunately, a phase II clinical trial showed no effects of UDCA in reducing liver volume in patients with this disease (D'Agnolo et al. 2016). Clinical data on NorUDCA in alpha-1 antitrypsin deficiency is still unavailable.

5 Summary and Outlook

Significant advances have been made in the understanding of the beneficial effects of hydrophilic BA in the liver. Both basic science and clinical studies have either disclosed the mechanisms of action or proved the efficacy of compounds that were found to be medically useful hundreds of years ago. The science of BA will continue developing, and new evidence will likely provide foundations for new, evidence-based, and effective treatments for certain common and uncommon liver diseases.

Declaration of Interest The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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Chenodeoxycholic Acid: An Update on Its Therapeutic Applications

Stefano Fiorucci and Eleonora Distrutti

Contents

Abstract

Chenodeoxycholic acid (CDCA), 3α,7α-dihydroxy-5β-cholan-24-oic acid, is a primary bile acid generated in the liver from cholesterol. In liver cells CDCA is conjugated with glycine or taurine to form two bile salts, Glyco-CDCA and Tauro-CDCA, before being released into the bile ducts. In the intestine, CDCA

S. Fiorucci (✉)
Section of Gastroenterology, Department of Surgical and Biomedical Sciences, University of Perugia, Perugia, Italy

Perugia Medical School, Perugia, Italy
e-mail: Stefano.fiorucci@unipg.it

E. Distrutti
Azienda Ospedaliera di Perugia, Perugia, Italy

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is further metabolized to generate a 7β epimer, i.e., the ursodeoxycholic acid (UDCA), or dehydroxylate to generate lithocolic acid (LCA). In humans, CDCA is the physiological ligand for the bile acid sensor farnesoid X receptor (FXR), while LCA is a potent agonist for a G protein-coupled receptor, known as GPBAR1 (TGR5). Along with UDCA, CDCA has been clinically used for the dissolution of gallbladder stones at doses ranging from 375 to 750 mg/day, with a success rate of 8 to 18%. Because the efficacy of CDCA was significantly lower than that of UDCA and 18–30% of patients developed significant side effects, the most frequent being diarrhea and a reversible increase in aminotransferases plasma levels, this application has lost its therapeutic relevance. Additionally, the combination of CDCA with UDCA, generally at doses of 5–10 mg/kg each, has failed to provide significant advantages over UDCA alone. In 2017, CDCA has been approved as an orphan indication for the treatment of patients with cerebrotendinous xanthomatosis (CTX), a rare autosomal recessive disorder caused by mutations of sterol 27-hydroxylase (CYP27A1) gene. Since CYP27A1 is essential for cholesterol breakdown, CTX patients develop abnormal lipid storage with increased plasma and tissue levels of cholestanol and very low/absent production of CDCA. CDCA is a potent inhibitor of CYP27A1, and early initiation of CDCA therapy, at doses up to 750 mg/day, is considered the standard medical therapy for CTX resulting in decreased plasma levels of cholestanol and stabilization of neurologic symptoms. Studies in CTX patients have also shown that CDCA might suppress the activity of 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase in the liver. Furthermore, CDCA promotes the release of glucagon-like peptide-1 (GLP-1) in diabetic patients, likely by activating GPBAR1.

Keywords
Bile acids · CDCA repositioning · Cerebrotendinous xantomatosis · Clinical applications · FXR · Gallbladder stones · GPBAR1

1 Introduction

The therapeutic utility of bile acids was recognized almost 50 years ago, when CDCA first (Danzinger et al. 1972) and UDCA later (Makino et al. 1975) were shown effective in solubilizing cholesterol stones in the gallbladder. Since the early seventies of the twentieth century, CDCA and UDCA have been extensively used for almost two decades in the treatment of gallbladder stones, but their clinical application has declined thereafter after the advent of laparoscopic cholecystectomy in the middle of 1990s. Nowadays, gallbladder stones are removed by laparoscopic cholecystectomy and the role of bile acid therapies for gallstone dissolution has lost clinical relevance. In contrast to CDCA, however UDCA, a highly soluble and nontoxic bile acid, has gained clinical application in other liver disorders including treatment of primary biliary cholangitis (PBC) and primary sclerosing cholangitis

Table 1 Therapeutic applications of CDCA

Disease	Recommended dose	Remarks
Cholesterol gallstones dissolution	750 mg/day	Limited efficacy: 8–18% of patients achieved stone dissolution. Dose dependency
Cerebrotendinous xanthomatosis	250 mg TID	Effective in reducing plasma and tissue levels of cholestanol Improves neurological symptoms
Steroid dehydrogenase deficiency	10–15 mg/kg/day	Small pilot studies. Few or no effects in clinical settings
Constipation	350 mg TID	Small pilot study
Hyperlipoproteinemia	325 mg BID	Decrease in serum triglyceride levels in small pilot studies. May be useful in the treatment of type IV hyperlipidemic patient

(PSC) and other adult and pediatric cholestatic disorders, including drug-induced liver injury. The therapeutic applications of UDCA are reviewed by Arrese et al. (2019) in another chapter of this book. Table 1 describes some of the potential therapeutic applications of CDCA.

2 Bile Acid Metabolism: A Short Overview

Bile acids are synthesized in the liver from the breakdown of cholesterol, representing the end products of cholesterol metabolism (Li and Chiang 2014). In mammals all bile acids are C_{24}-5β-bile acids (cholanoic acid). The steroid core consists of three 6-carbon rings and one 5-carbon ring. The transformation of cholesterol into primary bile acids takes place in hepatocytes and involves 17 enzymes (Fig. 1) necessary for modifying the cholesterol steroid core (including the saturation of the double bond at C5–C6 and the epimerization of hydroxyl group in C3), the cleavage of a 3-carbon unit in the side chain to generate a C23 steroid and the amidation with glycine and taurine at the C23 position, resulting in two main steroids: cholic acid (CA) [3α,7α,12α-trihydroxy-5β-cholan-24-oic acid] and chenodeoxycholic acid (CDCA) [3α,7α-dihydroxy-5β-cholan-24-oic acid] (Li and Chiang 2014) (Fig. 2). The liver enzymes involved in bile acids formation are grouped into two major metabolic pathways named as "classical" and "alternative." The "classical" pathway of bile acid biosynthesis starts with the 7alpha-hydroxylation of cholesterol by cytochrome P450 cholesterol 7α-hydroxylase (CYP7A1) a microsomal enzyme which catalyzes the first and rate-limiting step in the classic pathway (Li and Chiang 2014). The 7-hydroxy-4-cholestene-3-one can be hydroxylated at C-12 position by microsomal sterol 12α-hydroxylase (CYP8B1), followed up by several reactions including mitochondrial 27-hydroxylase (CYP27A1) to cleave a 3-carbon unit and eventually converted to CA. Without 12α-hydroxylation, 7α-hydroxy-4-cholestene-3-one is converted to CDCA. Thus,

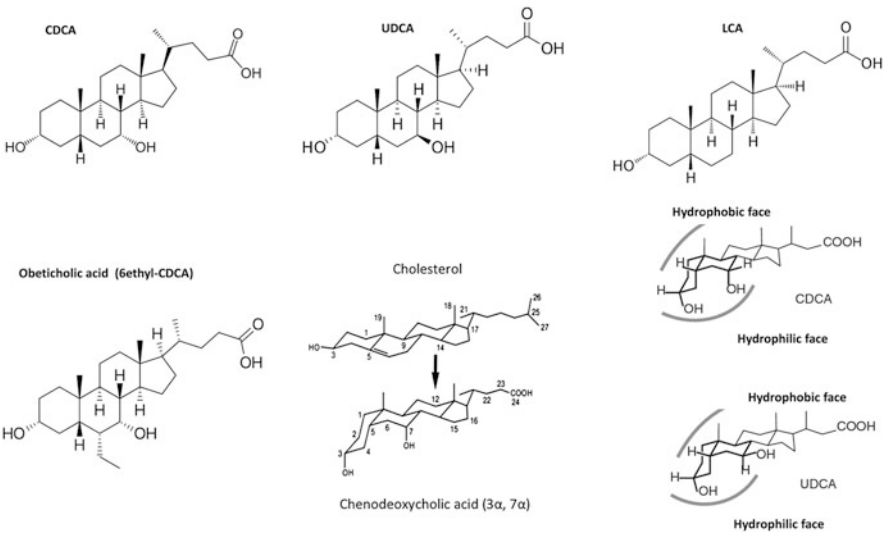

Fig. 1 Structure of chenodeoxycholic acid (CDCA), ursodeoxycholic acid (UDCA), lithocholic acid (LCA), and obeticholic acid (OCA). The hydrophobic and hydrophilic faces are shown

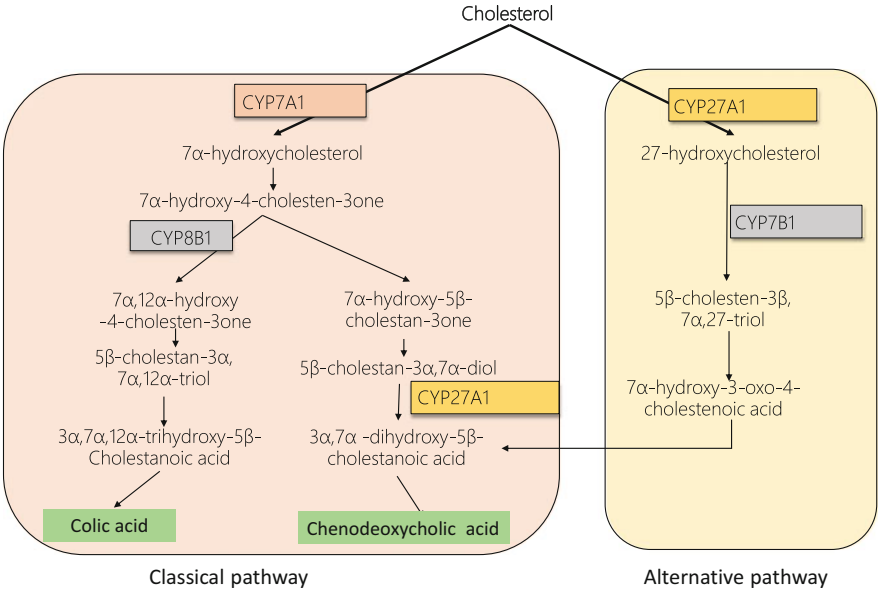

Fig. 2 Schematic representation of the classic and alternative pathways of bile acid synthesis. The classic pathway of bile acid synthesis is initiated from cholesterol by CYP7A1, which leads to the formation of 7α-hydroxycholesterol which, after some metabolic reactions, interacts with CYP8B1 or CYP27A1 to form cholic acid or chenodeoxycholic acid. The alternative pathway of bile acid synthesis begins with the metabolism of cholesterol to 27-hydroxycholesterol via CYP27A1. 27-Hydroxysterol is subsequently metabolized by CYP7B1 which, after a few metabolic steps, leads to the synthesis of CDCA

CYP7A1 controls the overall rate of bile acid production, while CYP8B1 controls the CA: CDCA ratio in the bile acid pool (Fig. 1). The classical pathway produces equal amounts of CA and CDCA, and is responsible for the large majority of CA and CDCA (90%) found in normal settings. The "alternative" pathway, also known as "acidic" pathway, starts with the C27 hydroxylation of cholesterol by the CYP27A1 followed by its conversion into 3β-dihydroxy-5-cholestionic acid. Central to this pathway is the 7α-hydroxylation of these two intermediates by the oxysterol 7-α-hydroxylase (CYP7B1), which are then converted into CDCA. This pathway only generates CDCA and its role increases in cholestatic settings (Li and Chiang 2014).

Before secretion by hepatocytes, in humans, bile acids are conjugated with glycine (70%) and taurine (this pathway is prevalent in rodents). The amidation processes are mediated by the activity of the two enzymes: the bile acid:CoA synthase (BACS) and the bile acid:amino acid transferase (BAT), and lead to the formation of the so-called bile salts, which are more hydrophilic of bile acids, thus facilitating their secretion from the apical pole of hepatocytes via the bile salt export pump (BSEP). Conjugated bile acids gradually flow through the intra- and extrahepatic bile ducts, reaching the gallbladder, where the bile is stored during the interdigestive periods, to be released into the duodenum in response to food intake (Li and Chiang 2014).

In the intestine, primary bile acids are metabolized by gut bacteria to secondary (or degenerated) bile acids, i.e., deoxycholic acid (DCA) and lithocholic acid (LCA), and to lesser extent, ursodeoxycholic acid (UDCA). Primary and secondary bile acids compose the total bile acid pool, in a relatively fixed proportion (Fiorucci and Distrutti 2015).

Bile acids are amphipathic molecules with detergent-like actions and are responsible for the absorption of lipid and lipid-soluble vitamins. Together with these nutrients, bile acids are reabsorbed at a rate of ~95% in the distal ileum, via a sodium-dependent process mediated by the apical sodium-dependent transporter (ASBT), expressed in the brush border of enterocytes (Dawson 2017; Kullak-Ublick et al. 2004). ASBT mediates the active uptake of bile acids across the luminal plasma membrane of the enterocytes, while the ileal bile acid binding protein (IBABP) facilitates their intracellular diffusion to the basolateral membrane, where bile acids are secreted into the portal circulation by the organic solute transporters (OSTα/β) (Dawson 2017; Kullak-Ublick et al. 2004). Bile acids are then transported back to the liver by the portal blood and the flow increases significantly after a meal. In the liver, bile acids are removed from the sinusoidal blood at the basolateral membrane of hepatocytes by the Na+/taurocholate cotransporting polypeptide (NTCP; for conjugated bile acids) and the organic anion transporting proteins (OATP2s; for the unconjugated bile acids), and, after amidation, are secreted back into biliary canaliculi by BSEP as well as by the canalicular conjugate export pump (MRP2). This cycle is known as enterohepatic circulation and takes place few times a day,

ensuring that 95% of bile acids are reabsorbed and returned to the liver, leaving only 5% of the total bile acids pool to be excreted in the feces. In humans, the bile pool is ~3 g and is mainly made of hydrophobic bile acid, i.e., CA, CDCA, and DCA in a proportion of 40:40:20. In contrast, LCA is excreted in the feces and only a small portion is circulated back to the liver to be sulfo-conjugated by sulfotransferase and eventually secreted into bile.

3 Regulation of Bile Acid Metabolism

Bile acid metabolism in the liver and intestine is tightly regulated by a variety of regulatory factors that have been reviewed in the books by Keitel et al. (2019), Cariello et al. (2019), and Shin and Wang (2019). The reader is redirected to these chapters. However, essential to the understanding of pharmacology of CDCA is the interaction of this bile acid with the farnesoid –X receptor (FXR) and G protein-coupled receptor GPBAR1 or TGR5. In the liver, the activity of the CYP7A1 is negatively regulated by the relative amount of primary bile acids. CDCA and CA repress the activity of CYP7A1, at least by two different mechanisms that are both mediated by FXR. The first mechanism of regulation of CYP7A1 by CDCA requires the transcription of a FXR-regulated gene, known as the small heterodimer partner (SHP). SHP (NR0B2) is an orphan nuclear receptor that maintains the dimerization and ligand-binding domain of other nuclear receptors but lacks a conserved DNA-binding domain (Seol et al. 1996). In mice and humans, the SHP gene is predominantly expressed in the gallbladder and liver, and at lower levels in the adrenal, pancreas, and gastrointestinal tract. Several factors have been identified that regulate transcription of SHP, including steroidogenic factor-1 (SF-1), liver receptor homolog-1 (LRH-1), FXR, c-jun, hepatocyte nuclear factor 4α (HNF4α), estrogen receptor-related receptor γ (ERRγ), E2A gene products (E47, E12 and E2/5), liver X receptor α (LXRα), estrogen receptor α (ERα), sterol regulatory element-binding protein 1c (SREBP-1c), adaptor protein (AP1) (Fiorucci et al. 2004), pregnane X receptor (PXR), the core circadian component CLOCK-BMAL1, peroxisome proliferator-activated receptor γ (PPARγ), and upstream stimulatory factor-1 (USF-1) (Zhang et al. 2011). Induction of SHP by FXR represses the expression of CYP7A1 by liver, by a mechanism that involves the disruption of HNF4α/LRH complex at the promoter of CYP7A1. SHP inactivates LRH-1 by forming a heterodimeric complex with the protein leading to promoter-specific repression of CYP7A1, thereby establishing an elaborate autoregulatory negative feedback loop (Goodwin et al. 2003) inside the hepatocytes. Several studies have demonstrated that CDCA (Ellis et al. 2003) is potent inducer of SHP gene expression and repressor of CYP7A1 via activation of FXR (Fig. 3). A second mechanism of regulation of CYP7A1 involves the fibroblasts growth factor (FGF)15 (19 is the human ortholog). Thus, in postprandial states (Fig. 3), bile acids flow through the small intestine activates FXR in ileal enterocytes to induce the expression and release of FGF15/19, an atypical FGF that functions as a hormone. FGF15/19 reach the liver through the

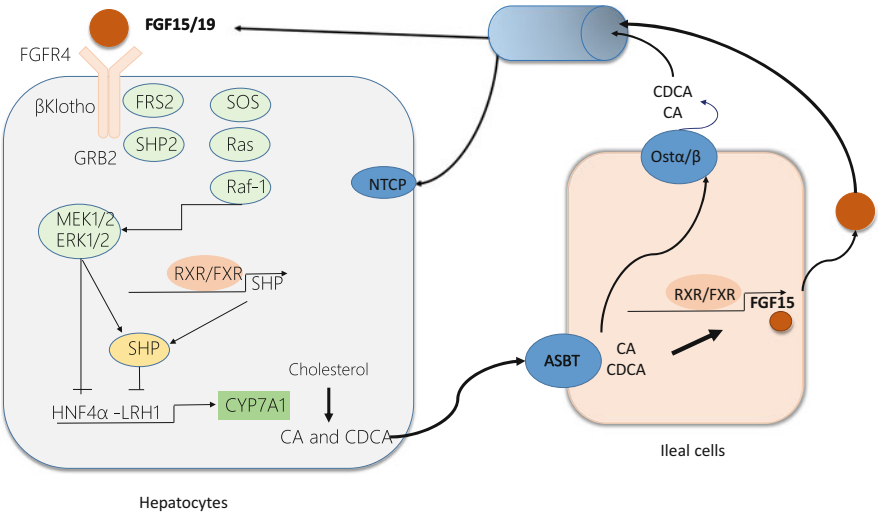

Fig. 3 Mechanism of regulation of bile acid synthesis by CYP7A1. Regulation of CYP7A1. In basal state, in liver cells the nuclear receptors HNF4α and LRH1 bind to a bile acid response element in the CYP7A1 gene promoter and stimulate CYP7A1 gene transcription. When bile acid concentrations increases, CA and CDCA activate FXR which is recruited to a RXR/FXR heterodimer in SHP promoter causing the transcription of the receptor. SHP interacts with HNF4α and LRH1, abrogating their binding to the CYP7A1 promoter. In the intestine, bile acid-activated FXR induces the transcription of FGF15 (FGF19 in humans), which is then released into the portal circulation and travels to the liver to bind and activate β-Kloto/FGFR4 complex on the hepatocytes. FGFR4 activates several intracellular signaling mechanisms including MEK1/2 and ERK1/2, which leads to the repression of CYP7A1 gene transcription

portal circulation and binds a specific receptor, FGF receptor 4 (FGFR4), on the surface of hepatocytes to repress CYp7A1 and bile acid synthesis (Inagaki et al. 2005; Moschetta 2019). Furthermore, FGF15 represses the gluconeogenesis and stimulates glycogen and protein synthesis. FGF15/19 also stimulates gallbladder filling. Thus, the bile acid-FXR-FGF15/19 signaling pathway regulates diverse aspects of the postprandial enterohepatic response (Owen et al. 2015; Fiorucci et al. 2018a, b).

4 CDCA for Cholesterol Gallstones

Despite CDCA is a relatively potent FXR ligand, its activity toward the receptor was discovered long after the clinical interest on this agent had vanished (Makishima et al. 1999; Parks et al. 1999; Wang et al. 1999). In fact, in the early days of its "therapeutic history," CDCA was used for its ability to dissolve cholesterol stones in the gallbladder, essentially for its ability to maintain cholesterol solubility in the bile. We know that this beneficial effect could be related, at least in part, to the activation of FXR (Moschetta et al. 2004), but in the 1970s and 1980s of the last century, the

receptor was still unknown and the beneficial effects of CDCA in this setting were explained on the basis of its physicochemical properties.

4.1 CDCA and the Critical Micellar Concentration

As mentioned above, CDCA is a hydrophobic molecule and this property is expressed by a measure known as critical micellar concentration (CMC). The CMC (Hofmann and Roda 1984) is a measure of the propensity of a specific bile acid to form micelles, and higher values of CMC indicate a lower potential for the formation of micelles and therefore the CMC is an indirect measure of its hydrophilicity (Natalini et al. 2014). The CMC depends on the amphipathic nature of bile acids and is due to the existence of a hydrophilic concave side (α-face) and a hydrophobic convex side (β-face). The number and position of the hydroxyl groups of bile acids directly determine the CMC value. The two hydroxyl groups of CDCA at positions C3 and C7 are in the α-configuration, and therefore the CMC of CDCA is lower than that of CA, which has three hydroxyl groups (C3, C7, and C12) in α-configuration. Bile acids, such as UDCA, with hydroxyl groups located on both sides of the hydrophobic steroid core (3α- and 7β-orientation) are more hydrophilic than any molecule with the same number of hydroxyl groups located in the α-orientation only. The relative hydrophilicity of different bile acids changes as follows: UDCA > CA > CDCA > DCA > LCA; taurine conjugates have higher CMC than glycine conjugates and free bile acids.

4.2 Mechanism of Action of CDCA in Gallstones Dissolution

In the Western world, prevalence of cholelithiasis has increased considerably over the twentieth century, paralleling the increased prevalence of overweight, obesity, diabetes, and metabolic syndrome. Overall, ~20% of adults in Western countries develop gallstones and 20% experience symptoms or ~1% complications. Approximately 70–80% of gallbladder stones are enriched in cholesterol (cholesterol content >50%), while less than 10% are made by bilirubin only (black stones). The prerequisite for gallstone formation is the precipitation of cholesterol to form crystals in a cholesterol-supersaturated bile (Lammert et al. 2016). Cholesterol is poorly soluble in an aqueous environment; however, in the gallbladder a relatively large amount of cholesterol is maintained in solution due to its incorporation in mixed micelles together with bile salts and phospholipids (mainly phosphatidylcholine). The main explanation for gallstone formation is that a supersaturation of bile by cholesterol and cholesterol crystallization occurs when either an excess of cholesterol is secreted into the bile or there is a relative decrease in solubilizing agents, i.e., bile salts and phosphatidylcholine. This establishes a correlation between the relative concentrations of these three agents in a disease model called “ternary phase model” (Carey and Small 1978). In this model, excessive cholesterol secreted in the bile is maintained in solution as a function of relative concentrations of bile acids and

phospholipids. Cholesterol crystal crystallization occurs when the relative concentrations of three factors change.

The ternary phase model explains why gallstone formation in vivo occurs in the gallbladder. Indeed, because a significant net water absorption occurs during prolonged storage in the gallbladder during the interdigestive period, mixed cholesterol–phospholipid–bile salt micelles are increasingly formed, and because bile salt concentrations progressively exceed CMC required for micelle formation, this increases the propensity for cholesterol crystal nucleation. In, fact, since the solubilizing capacity of micelles for phospholipids is higher than that for cholesterol, phospholipids are progressively transferred to the micelles and remaining vesicles become progressively cholesterol supersaturated. This phenomenon gives rise to progressive cholesterol crystallization. Importantly, more hydrophobic bile acids such as DCA promote faster cholesterol crystallization. On the contrary, hydrophilic bile acids such as UDCA (higher CMC) and, to less extent, CDCA (intermediate CMC) slow down the crystallization processes. Phospholipids also exert profound effects on cholesterol crystallization. The human bile is composed almost exclusively of phosphatidylcholine with unsaturated acyl chains, thus contributing to human vulnerability for gallstone formation (Lammert et al. 2016). In addition, to this chemical factor, the time to reach equilibrium is essential for human gallstone formation, since bile is stored in the gallbladder only for a limited period of time. For this reason, gallbladder stones occur in those settings that associate with impaired gallbladder emptying such as pregnancy, prolonged fasting or rapid weight loss due to intensive caloric restriction, diabetes, autonomic neuropathy, or pharmacological therapies (Fiorucci et al. 1992a, b; Fiorucci et al. 1990).

The mechanisms through which CDCA promotes litholysis are still unclear. It was originally hypothesized that CDCA effectively reduces the biliary secretion of cholesterol both in the meal-stimulated and in the fasted state. This effect is apparently linked to a decreased hepatic cholesterol synthesis, due to inhibition of 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase meva1onate: NADP oxidoreductase), a rate-limiting step in cholesterol biosynthesis. The expression of this enzyme is suppressed by CDCA (see below) leading to the formation of unsaturated bile thus preventing cholesterol crystals deposition and gallstones formation. In addition, CDCA, being relatively more hydrophilic than secondary bile acids, slows down the crystallization of cholesterol in the gallbladder. Finally, as mentioned above, CDCA is an FXR agonist, and animal studies have suggested a potential role for this receptor in gallbladder stone formation (Moschetta et al. 2004). FXR increases the bile flow and bile acid secretion, and its activation by CDCA might have beneficial effects on the cholesterol:bile acid:phospholipids equilibrium (Fig. 3). Interestingly, however, while FXR knockout mice are highly susceptible to a lithogenic diet, gene polymorphisms analysis of FXR in gallstone patients has been inconclusive.

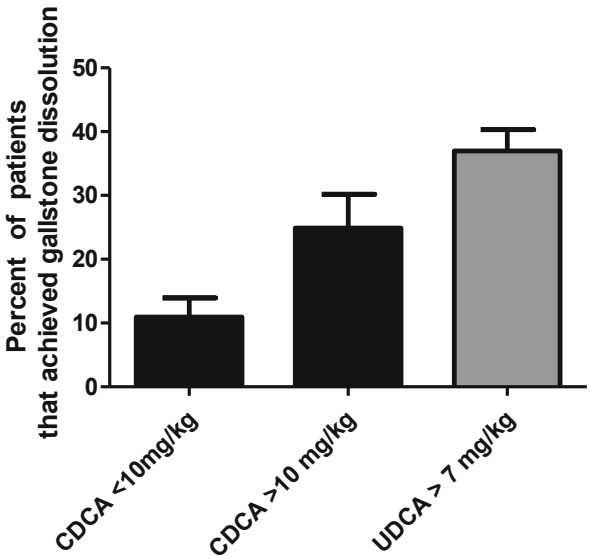

Fig. 4 Efficacy of bile acid therapy for gallstone dissolution. (Drawn from May et al. 1993)

4.3 Efficacy of CDCA in Promoting Gallstones Dissolution in Clinical Trials

Several studies have investigated the effect exerted by different doses of CDCA on gallbladder stones dissolution. In a meta-analysis, published in 1993, May et al. reported pooled data from 11 studies involving 1,062 patients, and concluded that complete gallstone dissolution occurred overall in 13.6% (95% C.I. 12–16%) of patients treated with CDCA. The trials were dishomogeneous, because, among other factors, CDCA was used at different doses. Subsequent sub-analysis of these studies allowed to identify trials in which patients were administered at a dose of CDCA $<$10 mg/kg or $<$750 mg/day (467 patients, low dose) and nine studies in which 589 patients were administered a higher dose ($>$10 mg/kg or $>$750 mg/day) for more than 6 months. The gallstone dissolution rate was 8.1% (95% C.I. 5.8–11%) with the low dose and 18.0% (95% C.I. 15–21%) with the higher doses (Fig. 4). High-dose CDCA dissolved significantly more stones than low-dose CDCA ($x' = 20.7$, $P < -0.001$). The results of this meta-analysis were largely confirmatory of the disappointing results obtained in the largest of these trials, the National Cooperative Gallstone Study published in 1981 (Schoenfield and Lachin 1981), that reported a dissolution rate of 13.2% in patients administered 750 mg/day and 5.2% in patients taking 375 mg/day. Side effects including clinically significant hepatotoxicity occurred in 3% of patients treated with 750 mg/day and were always reversible biochemically. Elevations of 10% or more of serum cholesterol, mostly LDL, occurred in 85.2% of patients treated with the higher dose.

In addition to a stand-alone therapy, CDCA has been used in combination with UDCA. Several combinations of doses have been investigated; however, a dose of

7 mg/kg UDCA and 5 mg/kg CDCA has been popular for several years (Petroni et al. 1995). While only few studies are available, it might be estimated that a complete gallstone dissolution occurs in approximately 60% of patients using this combination. This percentage was confirmed by the results of a recent trial carried out in Korea (Lee et al. 2015), which estimated a dissolution rate of 41.5% in the UDCA group and 56.5% in the UDCA+CDCA group ($P = 0.13$), suggesting that combining CDCA with UDCA achieves better dissolution rates than each agent alone. Other trials have confirmed this finding, including Hyun et al. (2015). In this later study, patients were given a magnesium trihydrate formulation of UDCA and CDCA for 6 months; the dissolution rate was 45.1% and the response rate was 47.2% (92/195) after 6 months of therapy. Only the stone diameter was significantly associated with the response rate. Both the symptom score and the number of patients with symptoms significantly decreased regardless of stone dissolution. Adverse events necessitating discontinuation of the drug, surgery, or endoscopic management occurred in 2.5% (6/237) of patients. In summary, these studies suggest that CDCA is significantly less effective than UDCA in promoting gallstone dissolution, although CDCA might exert some additional beneficial effects when combined with UDCA.

4.4 Safety of CDCA in Gallstone Patients

The therapeutic use of CDCA as a gallstones dissolution therapy has been associated with liver injury. In the National Cooperative Gallstone Study conducted on 916 patients treated with two doses of CDCA, 375 mg/day and 750 mg/day for over a 2-year period (Schoenfield and Lachin 1981), increased AST plasma levels occurred in 31% of patients who received 750 mg/day and 18% in patients who received 375 mg/day CDCA, and among them 3% of patients presented clinically significant hepatotoxicity that required the termination of CDCA. Additionally, diarrhea occurred in 41% of patients treated with the dose of 750 mg/day ($P < 0.05$ in comparison to placebo) and 20% in those treated with 375 mg/day of CDCA (not significant versus placebo). Safety of the use beyond 24 months was not established in clinical trials. Serum aminotransferase levels should be carefully monitored at regular intervals in patients taking CDCA. In the final product label, CDCA was given a boxed warning for its potential liver toxicity and is only recommended as therapy for treating patients with radiolucent gallstones, preferably in patients who have floatable or small gall stones and when surgery for removing the gallstones is too risky.

5 Cerebrotendinous Xanthomatosis

The cerebrotendinous xanthomatosis (CTX) is a rare autosomal recessive inborn disorder of bile acid metabolism due to mutations in the CYP27A1 gene, leading to damage to multiple tissues including the CNS. CTX was first reported by Van

Bogaert et al. in 1937 (Björkhem 2013), and since then several hundred patients have been described. The clinical manifestations of the disease are caused by the accumulation of cholesterol and cholestanol, the 5α-saturated analog of cholesterol in tissues. In contrast to other cholesterol disorders, CTX patients exhibit normal blood cholesterol levels, but tissue concentrations of cholesterol/cholestanol are markedly increased. The xanthomatous lesions contain high levels of cholesterol and cholestanol. Although epidemiologic data are limited, there is a robust evidence that CTX may be substantially underdiagnosed. Indeed, the prevalence of CTX is estimated to be 3–5:100,000 individuals, but only few hundreds of cases have been reported worldwide to date (Nie et al. 2014; Bjorkem and Hansson 2010; Einarsson et al. 2001).

CTX is an autosomal recessive disorder caused by the mutations in the CYP27A1 gene leading to decreased activity of the sterol 27-hydroxylase. The CYP27A1 is a ubiquitously expressed mitochondrial enzyme responsible for catalyzing multiple hydroxylation reactions in cholesterol metabolism and bile acid synthesis. As shown in Fig. 1, CYP27A1 catalyzes the first step in the alternative bile acid synthetic pathway and, in addition, the 27-hydroxylation of bile acid intermediates in the classic pathway. A reduced activity of CYP27A1 activity, in combination with a limited ability to cleave the cholesterol side chain by the alternative microsomal C-25 hydroxylation pathway, results in diminished CDCA (Cali et al. 1991) This leads to accumulation of bile acid intermediates with incompletely oxidized side chains, in particular bile alcohols with a hydroxyl group at the 25-position that will be excreted as glucuronides in the urine. In addition, upregulation of cholesterol synthesis and enhanced production of cholestanol (the 5α-dihydro derivative of cholesterol) result in increased plasma cholestanol levels and accumulation of cholestanol and cholesterol in tissues throughout the body, but especially in the CNS, lens, and tendons (Nie et al. 2014), leading to the development of neurologic symptoms cataracts and tendon xanthomas (Nie et al. 2014). CTX present usually with a multi-organ involvement and a broad range of neurological and nonneurological symptoms including intractable infantile-onset diarrhea and psychomotor retardation. The mean age at onset of symptoms in patients with CTX is 19 years, but the average age at the time of diagnosis is 35 years (range 23–44) (Nie et al. 2014).

A fairly large number of different mutations of CYP27A1 have been detected in CTX patients, including 11 missense and 10 null mutations (3 nonsense, 4 minor insertions/deletions, and 3 splice junction mutations). Various mutations in all nine exons and in introns 2,4,6,7, and 8 of the CYP27A1 gene have been described worldwide (Nie et al. 2014) Fifty percent of mutations in CYP27A1 have been detected in the region of exons 6–8, 16% in exon 2, and 14% in exon 4. Some of these missense mutations map to the adrenodoxin-binding site or to the heme ligand-binding region of the protein, i.e., two regions that are deemed essential for cofactor binding, and are crucial for the function of the enzyme, as well as for other members of the cytochrome P-450 family (The Human Gene Mutation Database at the Institute of Medical Genetics in Cardiff 2017).

The mutation of CYP27A1 causes cholesterol/cholestanol accumulation by different mechanisms: (1) increased synthesis of cholesterol in the liver. This effect could be secondary to the reduced formation of bile acids, in particular CDCA, leading to a reduced feedback on CYP7A1 in hepatocytes, an effect that is mediated by FXR (Fig. 3). Increased synthesis of cholesterol might also be the result of the lack of 27-hydroxycholesterol, which is known to be a potent inhibitor of HMG CoA reductase. However, the finding that extrahepatic cells from patients with CTX do not appear to have increased synthesis of cholesterol does not fit with this hypothesis. In any case, in patients with CTX, cholestanol accounts for up to 10% of sterols in xanthomas, 10% in bile, and up to 50% in the brain (Duell et al. 2018).

The biochemistry of CTX patients is characterized by high plasma cholestanol concentrations, five- to tenfold greater than normal (330 $\pm$ 30 μg/dL), increased urine and plasma bile alcohol concentrations, along with normal-to-low plasma cholesterol concentration, and decreased/absent CDCA levels.

In 2017, CDCA has been approved by the European Union (EU) as an orphan drug for the treatment of CTX in infants ($\geq$1 month), children, adolescents, and adults (European Medicines Agency 2017). The recommended doses are: 5–15 mg/kg/day for pediatric patients with lower doses preferred in younger children and 750 mg/day for adult patients (Berginer et al. 1984; Dotti et al. 2004; Ginanneschi et al. 2013; Martini et al. 2013; Huidekoper et al. 2016). CDCA is a life-long therapy and should be monitored for side effects. CDCA exerts several beneficial effects in CTX patients. This treatment has an inhibitory effect on CYP7A1, with a reduced accumulation of intermediates in bile acid biosynthesis. Indeed, suppression of CYP7A1 reduces the production of cholestanol and normalizes the levels of 7-α-hydroxy-4-cholesten-3-one, a bile acid precursor capable of efficient transfer across the blood–brain barrier and is converted to cholestanol in neuronal glial cells. CDCA also reduces the susceptibility of LDL to oxidative modification and enhances cholesteryl ester transfer protein (CETP) activity. As mentioned above, CDCA might also reduce the activity of HMG CoA reductase, thereby suppressing cholesterol production and xanthoma formation. CDCA is effective in improving biochemical abnormalities including the reduction of cholestanol and other sterols (e.g., lanosterol, campesterol, and sitosterol) and bile acid precursors (e.g., 7-α-hydroxy-4-cholesten-3-one) (Berginer et al. 1984) although it does not significantly reduce tendon xanthomas formation or improve cataracts. Several studies have noted, however, that CDCA stabilize or improve neurologic manifestations, including cognitive deterioration.

Assessment of plasma cholestanol may be useful in monitoring patient adherence to treatment. In addition, 7α-hydroxy-4-cholesten-3-one quantification may be a more sensitive indicator of response to treatment such that it may be reasonable to follow those levels annually.

Although CDCA supplementation is highly effective in reducing cholestanol concentrations in patients with CTX, mortality can still be relatively high in those undergoing CDCA supplementation. Therefore, other treatment strategies may be necessary to improve outcomes in patients with CTX. Alternative treatments have been examined, including cholestyramine clofibrate statins, alone or in combination

with CDCA but have generally shown limited efficacy in reducing and maintaining reduced cholestanol levels and/or demonstrating significant clinical improvement (Berginer et al. 1984; Ito et al. 2003; Dotti et al. 2004; Ginanneschi et al. 2013; Martini et al. 2013; Huidekoper et al. 2016).

5.1 Safety of CDCA in CTX Patients

Few specific adverse events or safety concerns have been reported for CTX patients treated with CDCA, with several reports indicating no adverse events (Berginer et al. 1984; Nie et al. 2014).

6 Additional Clinical Applications of CDCA

In addition to the two indications mentioned above, CDCA has been used as ancillary therapy for few other clinical applications.

6.1 CDCA and 11β-Hydroxysteroid Dehydrogenases (11β-HSDs)

The 11beta-hydroxysteroid dehydrogenase (11beta-HSD) metabolizes active glucocorticoids to their inactive 11-dehydro products and protects renal mineralocorticoid receptors from the high circulating levels of endogenous glucocorticoids. The enzymes might be important not only in the control of renal sodium retention but also in regulating blood pressure. Indeed, several studies have shown a role for the 11β-HSD1 in the pathogenesis of the metabolic syndrome, diabetes mellitus, and visceral obesity (Diederich et al. 2000). CDCA is a putative candidate for selective inhibition of human 11β-HSD1 (Diederich et al. 2011), but the clinical relevance of these observations remain unproved.

6.2 Regulation of Cholesterol and Bile Acid Metabolism

Few studies have examined the potential of CDCA in the regulation of cholesterol metabolism. Studies carried out in CTX patients have shown that CDCA might suppress the activity/expression of the hepatic 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase, the rate-limiting enzyme in cholesterol synthesis. Early studies (Adler et al. 1975; Ahlberg et al. 1981; Einarsson and Grundy 1980) have shown that feeding healthy subjects with CA, CDCA or DCA impact on cholesterol metabolism for 3 weeks in a randomized crossover study impact on cholesterol and bile acid synthesis. In 1981, Einarsson et al. have reported that treating healthy subjects with DCA and CDCA reduces cholesterol biosynthesis. By measuring the serum levels of 7alpha-hydroxy-4-cholesten-3-one, a measure of cholesterol 7alpha-hydroxylase activity, and 7-dehydrocholesterol, as a measure for

the HMG CoA reductase activity, and bile acids, these authors have shown that CDCA and DCA decreased the serum levels of 7alpha-hydroxy-4-cholesten-3-one by 80% and 75%, respectively. Negative correlations between the percentages of CDCA and DCA and the serum concentration of 7alpha-hydroxy-4-cholesten-3-one were observed. CDCA reduced the serum level of 7-dehydrocholesterol by 29%, whereas treatment with DCA tended to increase the level of 7-dehydrocholesterol.

These findings have not been confirmed by others. Indeed, a small pilot study by Wang et al. (2006) that included 11 subjects administered 15 mg/kg/day CDCA or no bile acid for 20 days while being fed a controlled diet, demonstrated that CDCA supplementation had no effect on cholesterol absorption and plasma lipid concentrations. CDCA treatment enriched ($P < 0.0001$) bile with CDCA and increased cholesterol concentration in micelles, whereas meal-stimulated bile acid concentrations were decreased. In contrast to CDCA, treatment with DCA effectively reduced plasma high-density lipoprotein (HDL) cholesterol. Taken together, these results suggest that the effect of CDCA on cholesterol metabolism is relatively modest. Confirming this view, obeticholic acid, a semisynthetic derivative of CDCA, exerts minimal impact on cholesterol plasma levels.

6.3 Regulation of GLP-1 Secretion After Bariatric Surgeries

The glucagon-like peptide-1 (GLP-1) has attracted a wide attention in recent years for its therapeutic potential in the treatment of diabetes. GLP-1 is released from intestinal endocrine cells, L cells, located in the distal ileum. GLP-1 secretion from these cells is robustly regulated by secondary bile acids, DCA and LCA, mostly acting on a cell membrane receptor known as GPBAR1. Along with other intestinal hormones, such as glucagon, neurotensin, and peptide YY (PYY), increased levels of GLP-1 occur in patients undergoing bariatric surgeries for morbid obesity. Because CDCA enhances postprandial levels of GLP-1, neurotensin, and PYY, which might exert synergistic effects in suppressing appetite, it has been suggested that this bile acid might have utility in modulating the hormonal response to food in the setting of obesity surgeries (Nielsen et al. 2017; Meyer-Gerspach et al. 2013). While only few clinical trials are available, it has been demonstrated that treating patients who underwent Roux-en-Y gastric bypass (RYGB) with CDCA increases postprandial levels of GLP-1. In a small pilot study, involving 11 subjects with RYGB administration of a single dose of 1,250 mg CDCA, increased fasting plasma concentrations of GLP-1, C-peptide, glucagon, peptide YY, neurotensin, total bile acids, and fibroblast growth factor 19 were significantly compared with placebo. These results were consistent with the results obtained in healthy volunteers (Meyer-Gerspach et al. 2013) and nonobese diabetic patients (Hansen et al. 2016), and strongly suggest a potential therapeutic utility of CDCA in ameliorating glycemic control in obese patients after bariatric surgery.

7 Conclusions

With the notable exception of the cerebrotendinous xanthomatosis, the clinical use of CDCA has greatly declined over the years. In the current clinical practice, the use of oral bile acids for cholesterol gallstone dissolution is limited to patients who are at risk for laparoscopic cholecystectomy, and UDCA has been shown to be more effective than CDCA alone in reaching complete stone dissolution. Furthermore, the fact that exposure to CDCA alters aminotransferase plasma levels in a significant proportion of patients appears to prevent further development of CDCA as a therapeutic agent. The use of CDCA has witnessed a partial revival in recent years because of its activity toward FXR. However, the advent of potent and selective FXR ligands in the therapeutic scenario do not allow tomajor therapeutic developments for CDCA in this very crowd arena.

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Obeticholic Acid: An Update of Its Pharmacological Activities in Liver Disorders

Stefano Fiorucci, Cristina Di Giorgio, and Eleonora Distrutti

Contents

Abstract

Obeticholic acid (OCA), 6α-ethyl-3α,7α-dihydroxy-5-cholan-24-oic acid, is a semisynthetic derivative of the chenodeoxycholic acid (CDCA, 3α,7-α-dihydroxy-5-cholan-24-oic acid), a relatively hydrophobic primary bile acid synthesized in the liver from cholesterol. OCA, also known as 6-ethyl-CDCA or INT-747, was originally described by investigators at the Perugia

S. Fiorucci (✉)
Section of Gastroenterology, Department of Surgical and Biomedical Sciences, University of Perugia, Perugia, Italy

Perugia Medical School, Perugia, Italy
e-mail: Stefano.fiorucci@unipg.it

C. Di Giorgio
Section of Gastroenterology, Department of Surgical and Biomedical Sciences, University of Perugia, Perugia, Italy

E. Distrutti
Azienda Ospedaliera di Perugia, Perugia, Italy

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University in 2002 as a selective ligand for the bile acid sensor, farnesoid-X-receptor (FXR). In addition to FXR and similarly to CDCA, OCA also activates GPBAR1/TGR5, a cell membrane G protein-coupled receptor for secondary bile acids. In 2016, based on the results of phase II studies showing efficacy in reducing the plasma levels of alkaline phosphatase, a surrogate biomarker for disease progression in primary biliary cholangitis (PBC), OCA has gained approval as a second-line treatment for PBC patients nonresponsive to UDCA. The use of OCA in PBC patients associates with several side effects, the most common of which is pruritus, whose incidence is dose-dependent and is extremely high when this agent is used as a monotherapy. Additionally, the use of OCA associates with the increased risk for the development of liver failure in cirrhotic PBC patients. Currently, OCA is investigated for its potential in the treatment of nonalcoholic steatohepatitis (NASH). Phase II and III trials have shown that OCA might attenuate the severity of liver fibrosis in patients with NASH, but it has no efficacy in reversing the steatotic component of the disease, while reduces the circulating levels of HDL-C and increases LDL-C. In summary, OCA has been the first-in-class of FXR ligands advanced to a clinical stage and is now entering its third decade of life, highlighting the potential benefits and risk linked to FXR-targeted therapies.

Keywords
FXR · GPBAR1 · Nonalcoholic steatohepatitis (NASH) · Obeticholic acid · Primary biliary cholangitis (PBC) · Side effects

1 Introduction

The farnesoid-X-receptor (FXR) is a member of the nuclear receptor superfamily. The receptor was originally described by Barry Forman et al. in 1995 while working at the Salk Institute in La Jolla, California (Forman et al. 1995). FXR was originally identified as a putative sensor for farnesol, an intermediate in cholesterol metabolism, and included in the growing family of nuclear receptors acting as sensors in the regulation of cholesterol metabolism. This family eventually expanded to more than 50 members that were grouped according to their structure in several subfamilies (Chawla et al. 2001). Similar to other members of nuclear receptor superfamily, FXR was shown to bind FXR-responsive elements in target genes, as a heterodimer with the retinoid-x-receptor (RXR), and to induce the transcription of these genes by recruiting co-activating factors and displacing co-repressors.

In 1999, three different groups reported that, rather than farnesol, bile acids should have been considered the physiologic ligands for FXR and identified chenodeoxycholic acid (CDCA, 3α, 7α-dihydroxy-5β-cholan-24-oic acid) as the most potent of endogenous ligands for the receptor. In addition to CDCA, the cholic

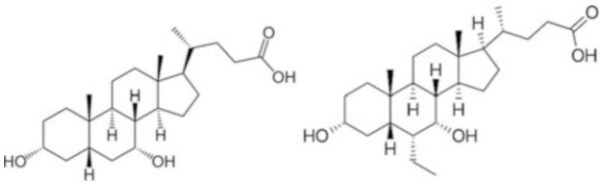

Fig. 1 Structure of CDCA and obeticholic acid. CDCA (3α,7α-dihydroxy-5-cholan-24-oic acid) and OCA (6α-ethyl-3α,7α-dihydroxy-5-cholan-24-oic acid)

acid (CA, 3α,7α,12α-trihydroxy-5β-cholan-24-oic acid), deoxycholic acid (DCA, 3α,12α-dihydroxy-5β-cholan-24-oic acid), and lithocholic acid (LCA, 3α-hydroxy-5β-cholan-24-oic acid) were also shown effective in activating human FXR, though at higher concentrations (Makishima et al. 1999; Parks et al. 1999; Wang et al. 1999).

Immediately after this discovery, the first synthetic ligand, GW4064, was identified by Maloney et al. (2000), at the Nuclear Receptor Discovery Research unit at the GlaxoSmithKline in North Carolina. GW4064 is a potent and selective nonsteroidal ligand for FXR (reviewed by Zampella 2019), and the use of this agent as a chemical tool allowed to rapidly progress in the identification of FXR pathways. Along with the generation of $FXR^{-/-}$ mice by the Gonzalez's group at NIH (Sinal et al. 2000), reported in 2000, these tools allowed seminal discoveries in the field of bile acid physiology.

Following these exiting progress, in 2002, in a paper published on *Journal of Medicinal Chemistry*, one of the authors of this manuscript (Fiorucci S) and other investigators of the University of Perugia, Italy, reported the discovery of a semi-synthetic ligand for FXR. This ligand was obtained by adding an ethyl group at position 6 on the B ring of CDCA (Fig. 1). The novel compound was 6α-ethyl-3α,7α-dihydroxy-5-cholan-24-oic acid, shortened as "6-ethyl-CDCA" (Pellicciari et al. 2002).

Shortly after this discovery, two of the authors of the *Journal of Medicinal Chemistry* paper, Fiorucci and Pellicciari, cofounded a start-up company, Intercept Pharmaceuticals, that took place in the development of the 6-ECDCA and as such (or INT-747) was used for several years in preclinical models of liver and intestinal disorders by the Fiorucci's group at the University of Perugia (Fiorucci et al. 2004, 2005a, b). In 2010, the name of the compound was changed again to obeticholic acid (OCA).

2 Obeticholic Acid and FXR and GPBAR1

The OCA is a potent FXR ligand, being able to activate the receptor with an EC_{50} that is the nanomolar range (300–600 nM) in transactivation assay (i.e., using HepG2 cells transfected with several copies of the receptor), but is lower (~100 nM) in cell-free assays (Pellicciari et al. 2002). Because CDCA activated FXR at an EC_{50} of ~10 μM in the transactivation assay, OCA is approximately 16- to 33-folds more potent than the natural ligand CDCA that transactivated the receptor with an EC_{50} of 10 μM (De Marino et al. 2019). These relative potencies are likely of

minor relevance in vivo because of the profound PK difference between OCA and CDCA (Fiorucci and Distrutti 2019).

While OCA was described originally as a selective FXR ligand, it has been demonstrated later that it might also activate GPBAR1 (TGR5). The first evidence that OCA acts as a GPBAR1 ligand comes from Intercept's team (Rizzo et al. 2010). These authors reported that INT-747 (see above) transactivated GPBAR1 (TGR5) at EC_{50s} ranging from 0.5 to 8 μM, in HEK 293 cells over-expressing the receptor. When the results from AlphaScreen for FXR assay and FRET in HEK203 cells were compared, the relative EC_{50} for the activation of FXR and GPBAR1 by OCA were 0.1 and 0.5 μM, providing a selectivity toward the two receptors of only fivefold. Additionally, it was shown that OCA transactivates GPBAR1 (TGR5) with an EC_{50} that is very close to that of LCA, the most potent endogenous ligand (i.e., 6 μM LCA and 8 μM for OCA). In a FRET analysis, the EC_{50} for cAMP formation (another measure of GPBAR1 agonism) were 0.3 μM for LCA and 0.5 μM for OCA. Together, these data indicate that OCA is dual FXR/GPBAR1 ligand with a modest selectivity toward the two receptors (Rizzo et al. 2010). These findings were confirmed later by D'Amore et al. who reported that OCA increases cAMP formation in HEK293 cells transfected with GPBAR1/TGR5 in the same range of concentrations of TLCA. OCA also releases GLP-1 from Glutag cells, a GPBAR1-mediated effect (D'Amore et al. 2014; Di Leva et al. 2013; Festa et al. 2014).

3 Efficacy of OCA in Liver Diseases in Animal Models

In the years 2002–2007, Fiorucci et al. carried out a large series of studies demonstrating that the 6-ethyl-CDCA-INT-747-OCA was effective in reversing or attenuating the severity of liver injury in several animal models. Some of these studies were instrumental to achieve the status of investigational new drug (IND) by FDA in 2006 (Fiorucci et al. 2011, 2004, 2014).

Due to the dual nature of OCA as an FXR and GPBAR1 ligand, it is likely that only a portion of the results reported in this study could be attributed to the activation of FXR alone.

4 Clinical Applications of OCA

Over the years we have reviewed the development of OCA several times, and therefore readers are redirected to the original papers and reviews (Fiorucci et al. 2011, 2014; Sepe et al. 2018). Additionally, excellent reviews on the applications of OCA in treating NASH are also included in this book (Guo 2019). To avoid redundancy, we will therefore focus the following part of this review only on clinical applications of OCA.

OCA has been approved in 2016 for the treatment of primary biliary cholangitis (PBC), and studies are ongoing for its potential in the treatment of patients with nonalcoholic steatohepatitis (NASH).

4.1 Use of OCA in PBC

4.1.1 OCA in UDCA-Resistant PBC Patients

In 2004, we demonstrated (Fiorucci et al. 2004) that OCA effectively increases bile flow in a rodent model of nonobstructive cholestasis caused by administering rats with estrogen. Following this line, OCA was proposed for the treatment of cholestasis in PBC, and was granted an orphan indication by NIH in 2006, and then was approved for clinical use in PBC patients, 10 years later.

Two phase II and one phase III studies were carried out to gain market approval.

In 2015, Hischfield et al. reported the results of a dose-finding study carried out in 165 PBC patients treated with increasing doses of OCA. All patients were considered to be resistant to treatment with UDCA. Inclusion criteria were as follow: patients should have been on a stable dose of UDCA for at least 6 months before screening, and PBC should have been diagnosed by at least 2 years by one of the following: history of increased alkaline phosphatase (Alk. Phos. or ALP) levels for at least 6 months; positive anti-mitochondrial antibody titer (>1:40 titer on immunofluorescence or M2 positive by enzyme-linked immunosorbent assay) or PBC-specific antinuclear antibodies; or liver biopsy consistent with PBC. Patients were required to have a mean baseline of ALP values between 1.5 and 10, the upper limit of normal range (upper normal limit = 117 U/L for women and 129 U/L for men) while taking UDCA. Key exclusion criteria were elevated plasma aspartate aminotransferase (AST) or alanine aminotransferase (ALT) levels, bilirubin >2 ULN; serum creatinine >1.5 mg/dL (133 mmol/L); use of colchicine, methotrexate, azathioprine, or systemic corticosteroids at any time during the 3 months before screening; and history of presence of hepatic decompensation. During the trial, patients maintained their initial dose of UDCA. A summary of the study results is shown in Table 1.

Results from a Phase III, the POISE study, Table 2, are also available (Nevens et al. 2016) and shown in Table 3.

In short, both studies demonstrated that OCA was effective in reducing ALP in UDCA-resistant patients. However, no evidence for a dose-dependency was identified. In contrast, side effects particularly pruritus were dose-dependent and their incidence increased significantly with the higher doses. As of today, OCA is the only drug for which controlled multicenter studies have demonstrated efficacy in the UDCA nonresponder or intolerant patient subgroup. OCA might be considered as a second-line therapy in UDCA nonresponders or intolerant PBC patients. However, pruritus occurs in a dose-dependent manner even at relatively low doses (5–10 mg/day) greatly limiting the use of this agent.

4.2 OCA as a Monotherapy in PBC

The results of Phase II study in which OCA was used as a monotherapy in PBC patients have been published recently. This study (Kowdley et al. 2018) reported data on safety, tolerability, and efficacy of OCA, 10 mg or 50 mg/day, administered

Table 1 Summary of original studies showing the efficacy of OCA (6-ethyl-CDCA or INT-747) in reversing/ameliorating liver injury in rodent models

Animal model	Dose tested	Remarks	Reference
Liver fibrosis			
Bile duct ligation	3 mg/kg	Attenuates liver fibrosis. Worsens cholestasis	Fiorucci et al. (2004)
Porcine serum	1–10 mg/kg	Attenuates liver fibrosis	Fiorucci et al. (2004)
CCL_4	3 mg/kg	Attenuates liver fibrosis	Fiorucci et al. (2005a, b)
Thioacetamide (TAA)		Attenuates liver fibrosis	Albanis et al. (2005), Verbeke et al. (2016)
Cholestasis			
Estrogen-induced cholestasis	3 mg/kg	Attenuates nonobstructive cholestasis	Fiorucci et al. (2005a, b)
BDL	3 mg/kg	Worsens obstructive cholestasis	Fiorucci et al. (2004)
Portal hypertension		Reduces portal pressure	
CCL_4	10 mg/kg		Renga et al. (2009)
BDL and TAA	30 mg/kg	Reduces portal pressure	Verbeke et al. (2014)
Immune activation			
Concanavalin A	10 mg/kg	Attenuates liver injury by a NKT mechanism	Mencarelli et al. (2009a, b)
NASH			
Genetic model Zucker rats	3 mg/kg/day	Ameliorates insulin sensitivity Attenuates NAS score	Cipriani et al. (2010)
Apo $E^{-/-}$	3–10 mg/kg/day	Reduces HDL No effect on cholesterol	Mencarelli et al. (2009a, b)

Table 2 Results of a phase II, dose-finding, study in UDCA-resistant PBC patients (Hirschfield et al. 2015)

Study harm (no. patients)	Daily dose of UDCA (mg/kg)	ALP (U/L)	Percent decreases, ALP value (%)	Percent of patients with pruritus. Any stage (severe pruritus)
Placebo (38)	15.9	275.2±102.7	3	50
OCA, 10 mg (38)	15.9	294.4±149.4	24	47 (16%)
OCA, 25 mg (48)	15.6	290.0±123.6	25	85 (24%)
OCA, 50 mg (41)	16.3	286.9±106.2	21	80 (37%)

Table 3 Results of a phase III trial (the POISE study-Nevens et al. 2016)

Study harm (no. patients)	Daily dose of UDCA at baseline (mg/kg)	ALP (U/L) baseline	Percent decreases of at least 15% ALP value (%)	Percent of patients with pruritus. Any stage
Placebo (73)	15 ± 4	327.2 ± 115	29	38
OCA 5–10 mg (70)	17 ± 5	326 ± 116	77	56
OCA 10 mg (73)	16 ± 5	316 ± 104	77	58

Table 4 Efficacy of OCA as a monotherapy in PBC patients (Kowdley et al. 2018)

Study harm (no. patients)	Daily dose of UDCA at baseline (mg/kg)	ALP (U/L) baseline	Percent decreases of at least 15% ALP value (%)	Percent of patients with pruritus. Any stage
Placebo (23)	NONE	321	1	35
OCA 10 mg (20)	NONE	366	53.9	70
OCA 50 mg (16)	NONE	379	37.2	94

as a monotherapy compared to placebo. The study also included data of an open-label extension of the Phase II trial for up to 6 years. The main results of phase II trial are shown in Table 4.

Again, pruritus was the most common adverse event in patients treated with OCA. The incidence of pruritus was as follows: placebo, 35%; OCA 10 mg, 70%; and OCA 50 mg, 94%. The incidence and severity of pruritus were dose-dependent with OCA since 15% of patients in the OCA 10 mg and 38% of patients in the OCA 50 mg discontinued the therapy because of the severity of pruritus. The median time for the onset of pruritus was 33, 14, and 6 days in the placebo, OCA 10 mg, and OCA 50 mg groups, respectively. In summary, the use of OCA as a monotherapy associates with severe pruritus in almost all the patients treated with 50 mg/day. No benefits over a co-therapy with UDCA were observed (further discussion on the role of FXR in cholestasis could be found in Haussinger and Keitel 2019).

4.3 Pharmacological Effects of OCA in PBC Patients: Effects on FGF19

FGF19 is a member of the fibroblast growth factor family released from the ileum in response to FXR activation by CA and CDA. Once released, FGF19 enters the portal circulation reaching the liver where it binds to and activate the FGFR4/βKlotho receptor on hepatocytes (Lang and Teng 2019; Potthoff et al. 2011). This activates a series of MAP-kinases (e.g., ERK-1 and ERK-2) and suppresses CYP7A1

expression (See Fiorucci and Distrutti 2019). Like FXR, FGF19 has strong metabolic effects as it suppresses the insulin-stimulated expression of the lipogenic enzymes such as fatty acid synthase (FAS) and Sterol Regulatory Element Binding Protein (SREBP)-1c in the liver and stimulates glycogen synthesis and reduces the expression of gluconeogenic enzymes (Kir et al. 2011; Potthoff et al. 2011). Unfortunately, activation of the FGF19/Bkloto complex has been associated with neoplastic proliferation in the liver, and the expression of the receptor is increased in patients with poorer prognosis and in unresectable liver carcinomas accordingly, inhibitor of FGF19/EGFR4 is currently developed for treating liver primary cancers. Increased levels of FGF19 occur in patients treated with OCA. Even the dose of 5 mg/day associates with significantly increased levels of FGF19 in PBC patients (Gao et al. 2018; Lin et al. 2019; Moschetta et al. 2019).

4.4 Postmarketing Surveillance in PBC Links OCA to Liver Failure and Death in Cirrhotic PBC

In 2017, after less than 2 years of marketing, a cluster of severe side effects including liver failure requiring intensive care therapy and liver transplantation linked to the use of OCA in cirrhotic PBC patients was reported. The searching of the FDA adverse Events Reporting System (FAERS) in March 2019 retrieves 3,200 side effects linked to the use of OCA in the years 2016, 2017, and 2018, 571 cases were considered serious side effects including 112 deaths. The severity of these effects has led the FDA to issue a drug safety communication on February 1, 2018, [source: FDA–Drug safety communication 02/01/2019] and a boxed warning was added stating that "Health care professionals should follow the Ocaliva dosing regimen in the drug label, which is based on calculating a Child-Pugh score in PBC patients with suspected liver cirrhosis before treatment to determine their specific classification and starting dosage (see Table for the Clarified Ocaliva Dosage Regimen and more detailed instructions). Dosing higher than recommended in the drug label can increase the risk for liver decompensation, liver failure, and sometimes death. Routinely monitor all patients for biochemical response, tolerability, and PBC progression, and re-evaluate Child-Pugh classification to determine if dosage adjustment is needed. Close monitoring is recommended for patients at an increased risk of liver decompensation, including those with laboratory evidence of worsening liver function (e.g., total bilirubin, INR, albumin) or progression to cirrhosis." The warning highlights that in patients with liver cirrhosis the initial dose of OCA should not exceed 5 mg once a week (keeping in mind that dose tested in Phase II and III trials were 5, 10, 25, and 50 mg/day).

4.5 OCA and NASH

Based on preclinical studies shown in Table 1, and its efficacy in reversing fibrosis, OCA has been tested for its efficacy in reducing liver fibrosis and steatosis in

Table 5 Efficacy and safety of OCA in type 2 diabetes (Mudaliar et al. 2013)

Analyte	Placebo (23)	OCA 25 mg/day (20)	OCA 50 mg/day (21)
FGF 19 (ng/L)	91 ± 11	177 ± 23[a]	255 ± 42[a]
Alk. Phos.	77 ± 21	86 ± 37[a]	103 ± 36[a]
LDL	107 ± 34	120 ± 31[a]	129 ± 35[a]
HDL	40 ± 11	35 ± 6	37 ± 7[a]
TG	1,788 ± 90	170 ± 81	156 ± 50[a]

[a]Denotes a statistically significant difference

patients with NASH (Fiorucci et al. 2018). Results from the two Phase II trials have been published (Mudaliar et al. 2013; Neuschwander-Tetri et al. 2015), while the results of a phase III trial (REGENERATE) are expected to be released later in 2019.

The results of the two Phase II trials are shown in Table 5.

The study included only a small number of patients but allowed to conclude that administration of 25 or 50 mg OCA for 6 weeks was well tolerated, increased insulin sensitivity, and reduced markers of liver inflammation and fibrosis in patients with type 2 diabetes mellitus and nonalcoholic fatty liver disease.

The FLINT study was RCT in which 283 patients with histologically proven NASH were randomly assigned to a treatment with placebo (142) or OCA 25 mg/day (141) for 72 weeks. From those patients who concluded the study and were eligible for analysis, 50 (45%) of 110 patients in the OCA group who were meant to have biopsies at baseline and 72 weeks had improved liver histology compared with 23 (21%) of 109 such patients in the placebo group (relative risk 2.2, 95% CI 1.4 to 3.3; $p = 0.0002$). Additionally, resolution of NASH was close to but did not reach a statistically significant value. Once again, while OCA reduced AST plasma levels, it increased Alk. Phos. value by ~15%. Treating patients with OCA associated with increased levels of total cholesterol, HDL-C, and LDL-C. No changes were observed on glucose plasma levels while insulin plasma levels and HOMA increased significantly.

Among the adverse events pruritus was the most common with 33 (23%) of 141 patients in the OCA group that developed pruritus compared with 9 (6%) of 142 in the placebo group. Pruritus was severe or widespread in 24 of 44 patients.

On February 2019, preliminary results on the Phase III REGENERATE study were made available. The REGENERATE study is a randomized, double-blind placebo-controlled multicenter trial evaluating the safety and efficacy of OCA among 931 people with Stage 2 or 3 liver fibrosis due to NASH. The participants were randomized into three even groups to receive a placebo, or OCA 10 mg/day OCA or 25 mg/day once daily for 18 months. Liver biopsies were performed at the end of the study. Pre-specified end points were: (1) a one-stage improvement in liver fibrosis with no worsening of NASH; or (2) a resolution of NASH with no worsening of fibrosis.

One-stage improvement of liver fibrosis with no worsening of NASH occurred in 11.9% in placebo harm and in 17.6% and 23.1% in patients treated with OCA 10 or 25 mg/day. The difference between the success rate of the placebo and the 25 mg dose of OCA was considered statistically significant. In the three groups, a respective 7.9

percent, 11.3 percent, and 14.9 percent experienced resolution of NASH with no worsening of liver fibrosis. Compared with the placebo, neither success rate of the two OCA doses was statistically significant.

Pruritus occurred in 19% of placebo, 28% of patients treated with OCA 10 mg/day, and 51% in patients treated with OCA 25 mg/day. The large majority of these cases were mild to moderate. Three percent of patients had gallstone and cholecystitis.

5 Conclusion

OCA is now approaching its third decade of life. In the last 20 years, this agent has been used extensively as a tool for decoding FXR activities in a number of biological and clinical settings (Vavassori et al. 2009). However, OCA is a relatively unselective dual FXR and GPBAR1 ligand. Some of the effects observed with OCA in vivo are likely linked to the activation of GPBAR1; however, when administered in animals or in clinical setting a robust induction biomarker of FXR activation is observed. Thus, there is no doubt that OCA is an FXR agonist in vivo.

In clinical settings, OCA has been associated with beneficial effects on surrogate biomarkers of disease progression in PBC patients. However, it remains to be determined whether the reduction in Alk. Phos. levels effectively correlates with the slowdown of disease progression and reduction of liver transplantation. Severe side effects have emerged when the drug has been dosed to cirrhotic PBC. This has required the addition of box warning by FDA and EMEA. Pruritus is dose-dependent and occurs almost at every dose. Importantly, the dose of 5–10 mg/day which is currently recommended for treating PBC patients associates with an increased risk of liver failure in cirrhotic patients. It is currently recommended that in cirrhotic PBC, therapy with OCA should begin with 5 mg/week. Importantly, most of the beneficial effects observed in Phase II trials in PBC were documented at doses of 25 and 50 mg/day, i.e., at doses that were significantly higher than that currently recommended.

The more recent history of OCA has been in NASH (Brunt et al. 2018; Hameed et al. 2018). In addition, to preclinical study, Phase II trials have demonstrated a potential benefit of OCA in treating fibrosis and steatosis in biopsy-proven NASH. In the FLINT study, patients were administered 25 mg/day OCA and pruritus occurred in the 25% of patients. In addition, changes in metabolic parameters including increased levels of cholesterol and LDL-C along with a reduced level of HDL-C have been noted. The results of a long awaited phase III trial (The REVERSE study) have been released in February 2019. It appears that only a fraction of patients had an improvement in liver fibrosis, and pruritus was again the most significant side effect occurring in a large proportion of patients taking the dose of 25 mg/day. These results indicate that OCA will be beneficial in a portion of patients with NASH, and that the quest for a therapy for NASH remains open.

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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

V. Keitel (✉) · C. Dröge · D. Häussinger
Clinic for Gastroenterology, Hepatology and Infectious Diseases, University Hospital Düsseldorf, Medical Faculty at Heinrich-Heine-University, Düsseldorf, Germany
e-mail: Verena.Keitel@med.uni-duesseldorf.de

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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

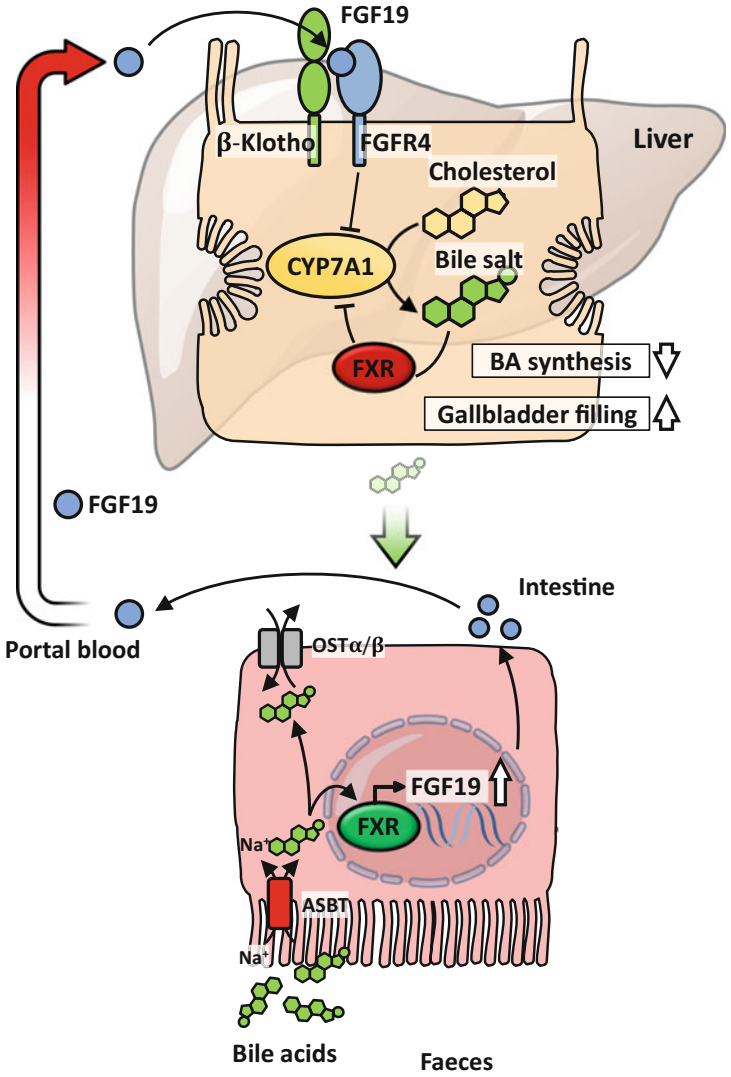

Fig. 1 FGF19 signaling in liver and intestine. Bile acids (BAs) are synthesized in the liver from cholesterol under control of CYP7A1. High hepatic BA concentrations activate FXR signaling thereby repressing CYP7A1 expression. In the intestine, FXR is activated by BAs resulting in increased FGF19 expression, 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).

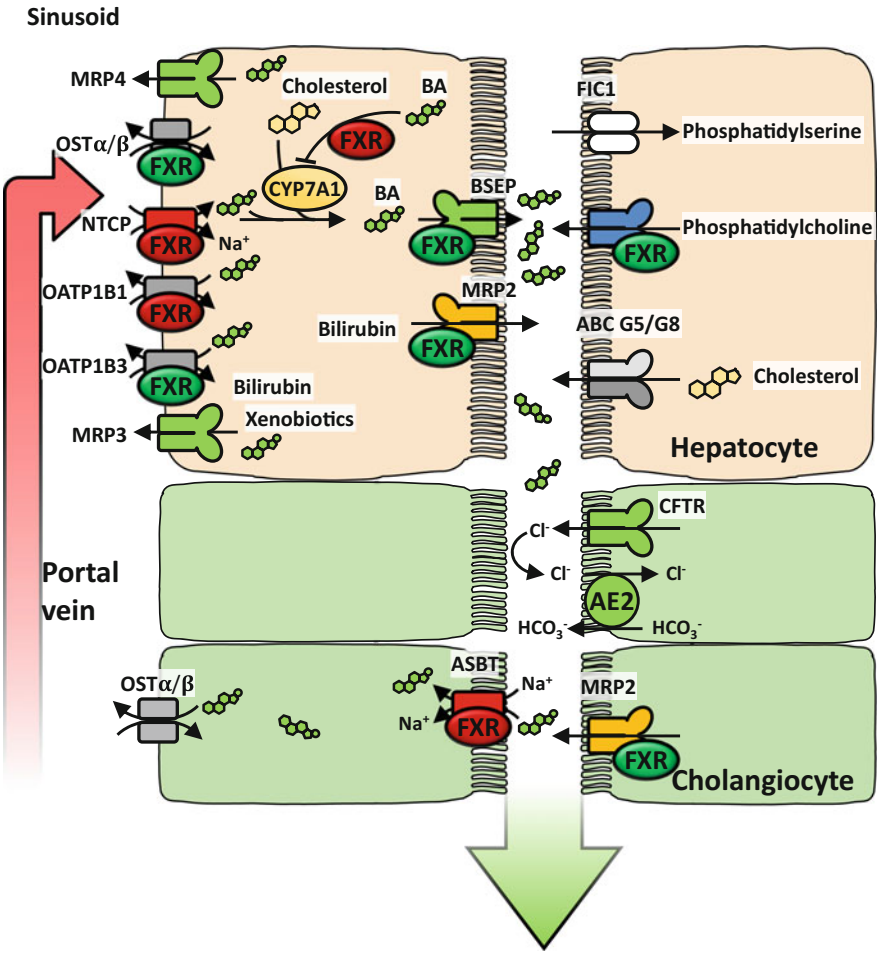

Fig. 2 Bile formation and regulation by FXR. Bile acids (BAs) are synthesized from cholesterol within hepatocytes and secreted by the bile salt export pump (BSEP) across the apical/canalicular membrane into the bile canaliculus. BAs form mixed micelles together with phosphatidylcholine transported by 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 cholangiocellular 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 cholestatic liver diseases.

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Pharmacologic Modulation of Bile Acid-FXR-FGF15/FGF19 Pathway for the Treatment of Nonalcoholic Steatohepatitis

Justin D. Schumacher and Grace L. Guo

Contents

Abstract

Nonalcoholic steatohepatitis (NASH) is within the spectrum of nonalcoholic fatty liver disease (NAFLD) and can progress to fibrosis, cirrhosis, and even hepatocellular carcinoma (HCC). The prevalence of NASH is rising and has become a large burden to the medical system worldwide. Unfortunately, despite its high prevalence and severe health consequences, there is currently no therapeutic agent approved to treat NASH. Therefore, the development of efficacious therapies is of utmost urgency and importance. Many molecular targets are currently under investigation for their ability to halt NASH progression. One of

J. D. Schumacher · G. L. Guo (✉)
Department of Pharmacology and Toxicology, Rutgers University, Piscataway, NJ, USA
e-mail: guo@eohsi.rutgers.edu

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the most promising and well-studied targets is the bile acid (BA)-activated nuclear receptor, farnesoid X receptor (FXR). In this chapter, the characteristics, etiology, and prevalence of NASH will be discussed. A brief introduction to FXR regulation of BA homeostasis will be described. However, for more details regarding FXR in BA homeostasis, please refer to previous chapters. In this chapter, the mechanisms by which tissue and cell type-specific FXR regulates NASH development will be discussed in detail. Several FXR agonists have reached later phase clinical trials for treatment of NASH. The progress of these compounds and summary of released data will be provided. Lastly, this chapter will address safety liabilities specific to the development of FXR agonists.

Keywords
Bile Acids · FGF15 · FGF19 · FXR · NASH

Abbreviations

αSMA	Alpha smooth muscle actin
βKL	Beta KLOTHO
AGRP	Agouti-related peptide
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
AP-1	Activator protein 1
ApoA-IV	Apolipoprotein A-IV
ApoC-III	Apolipoprotein C-III
ApoE	Apolipoprotein E
ASBT	Apical sodium-dependent bile acid transporter
BA	Bile acid
BBB	Blood-brain barrier
bFKB1	Bi-specific activating antibody of FGFR1 and β-Klotho
BSH	Bile salt hydrolase
C4	7α-hydroxy-4-cholesten-3-one
CA	Cholic acid
CAPE	Caffeic acid phenethyl ester
CCl_4	Carbon tetrachloride
CDCA	Chenodeoxycholic acid
COL1α1	Collagen type 1, α1
CREB	cAMP response element-binding protein
CRP	C-reactive protein
CTGF	Connective tissue growth factor
CYP27A1	Cytochrome P450 27A1
CYP7A1	Cytochrome P450 7A1
CYP8B1	Cytochrome P450 8B1
DCA	Deoxycholic acid
DDAH2	Dimethylarginine dimethylaminohydrolase 2

EETs	Epoxyeicosatrienoic acids
eNOS	Endothelial nitric oxide synthase
ERK	Extracellular signal-regulated kinases
ET-1	Endothelin-1
FGF15	Fibroblast growth factor 15
FGF19	Fibroblast growth factor 19
FGF21	Fibroblast growth factor 21
FGFR1	Fibroblast growth factor receptor 1
FGFR4	Fibroblast growth factor receptor 4
FXR	Farnesoid X receptor
FXRRE	Farnesoid X receptor response element
G6Pase	Glucose 6-phosphatase
GGT	γ-Glutamyltransferase
GLP1	Glucagon-like peptide-1
Gly-MCA	Glycine-conjugated muricholic acid
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HDL	High-density lipoprotein
HFD	High-fat diet
HOMA-IR	Homeostatic model assessment of β-cell function and insulin resistance
IκBα	Nuclear factor of kappa light polypeptide gene enhancer in B-cell inhibitor, alpha
ICV	Intracerebral-ventricular injection
IKKβ	Inhibitor of nuclear factor kappa-B kinase subunit beta
JNK	c-Jun N-terminal kinase
LCA	Lithocholic acid
LDL	Low-density lipoprotein
LDLR	Low-density lipoprotein receptor
LPS	Lipopolysaccharide
MCA	Muricholic acid
MCD	Methacholine-deficient diet
MCP-1	Macrophage chemoattractant protein 1
MMP2	Matrix metalloprotease 2
NAFLD	Nonalcoholic fatty liver disease
NAS	NAFLD activity score
NASH	Nonalcoholic steatohepatitis
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NKT	Natural killer T cell
NPY	Neuropeptide Y
OCA	Obeticholic acid
PBC	Primary biliary cirrhosis
PDC	Pyruvate dehydrogenase complex
PDK4	Pyruvate dehydrogenase kinase 4
PEPCK	Phosphoenolpyruvate carboxykinase

PGC1α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PIIINP	N-terminal propeptide of type III collagen
PNPLA3	Patatin-like phospholipase domain-containing protein 3
PPARα	Peroxisome proliferator-activated receptor alpha
PPARγ	Peroxisome proliferator-activated receptor gamma
Pro-C3	N-terminal type III collagen propeptide
RXR	Retinoid X receptor
SAA3	Serum amyloid A3
SAF	Steatosis, activity, and fibrosis scoring system
SAP	Serum amyloid P
SHP	Small heterodimer partner
SRB1	Scavenger receptor class B type 1
SREBP1c	Sterol regulatory element-binding protein 1c
TβMCA	Taurine-conjugated beta-muricholic acid
TCA	Taurocholic acid
TGFβ	Transforming growth factor beta
TGFβR2	Transforming growth factor beta receptor 2
TGR5	Takeda G-protein receptor 5
TIMP1	Tissue inhibitor of metalloproteases 1
TNFα	Tumor necrosis factor alpha
UCP1	Uncoupling protein 1
UDCA	Ursodeoxycholic acid
VLDL	Very low-density lipoprotein

1 Introduction: NASH

1.1 Disease Characteristics, Etiology, and Risk Factors

NASH is the inflammatory form of NAFLD characterized by steatosis, hepatocyte ballooning, inflammation, and fibrosis (Benedict and Zhang 2017; Hashimoto et al. 2013). NAFLD is a progressive disease beginning as simple steatosis but can develop into NASH that is characterized by inflammation and other cellular degenerations. NASH can further progress to fibrosis, cirrhosis, and even HCC (Benedict and Zhang 2017; Hashimoto et al. 2013; Michelotti et al. 2013). Metabolic syndrome often accompanies the development of NASH. Metabolic syndrome is defined as having three of five clinical presentations: (1) serum triglycerides greater than 150 mg/dL; (2) serum high-density lipoprotein (HDL) less than 40 or 50 mg/dL in men and women, respectively; (3) increase in waist circumference; (4) serum glucose levels greater than 100 mg/dL; and (5) systolic or diastolic blood pressures greater than 130 and 85 mmHg, respectively (Grundy et al. 2005).

The mechanisms regulating NAFLD to NASH progression remains unclear. A "two-hit" model was proposed in 1998 (Day and James 1998). This model speculates that NASH develops as the result of two sequential liver injuries. The "first hit" in the model being the accumulation of lipids in the liver leading to the

development of simple steatosis. The "second hit" is a subsequent insult that induces inflammation. Though this model has been well cited for two decades, it has come under recent scrutiny as it is likely a drastic oversimplification of the processes that lead to NASH. For instance, progression to fibrosis can occur in NAFLD without the development of NASH (Singh et al. 2015). Patients can also present with cryptogenic fibrosis and have numerous risk factors for NAFLD and NASH but have minimal histological features of NASH (Caldwell et al. 2009). Additionally, NASH patients can progress to HCC without the development of cirrhosis (Mittal et al. 2016). These findings indicate that more than just the "two-hit" model underlies disease pathogenesis.

Although the etiologies of NASH are not well understood, many risk factors have been identified. The most common health condition associated with NASH is obesity, followed by type 2 diabetes mellitus, dyslipidemia, metabolic syndrome, and polycystic ovary syndrome, while less common conditions include hypothyroidism, hypopituitarism, hypogonadism, pancreaticoduodenal resection, psoriasis, and sleep apnea (Chalasani et al. 2018). Age, sex, female reproductive status, and ethnicity are also associated with NASH development (Benedict and Zhang 2017). Lastly, genetic polymorphisms have been identified which correlate to NASH; the most notable being variation in the patatin-like phospholipase domain-containing protein (*PNPLA3*) gene (Dongiovanni et al. 2013; Romeo et al. 2008). The prevalence of *PNPLA3* polymorphisms among different ethnic groups may explain ethnic differences in NAFLD and NASH prevalence (Romeo et al. 2008).

1.2 Disease Prevalence, Diagnosis, and Current Treatment

With the rise of the obesity epidemic, the prevalence of NASH has greatly increased over the past two decades. Current estimates place the North American prevalence of NAFLD at 24% and of those patients with NAFLD 21% may have NASH (Younossi et al. 2016, 2018). Further, of NASH patients worldwide, 40% will likely progress to fibrosis. The US census data in 2017 placed the population at 325 million people (U.S. Census Bureau 2017). Based on the census and the estimates of NASH and fibrosis prevalence, we estimate that roughly seven million individuals in the United States alone have or will develop NASH with fibrosis. The high prevalence of NAFLD and NASH is not limited to North America with the global prevalence of NAFLD estimated at 25.24% (Younossi et al. 2016). Due to the increasing prevalence of NASH and recent breakthroughs in treatment of HCV, NASH will surpass HCV as the primary indication for which patients are added to the liver transplant waiting list. From 2008 to 2014, the number of patients added to the US transplant waiting list for the treatment of HCV was stable at roughly 3,000 patients per year (Organ Procurement and Transplantation Network n.d.). In 2017, this number was decreased to 1,705. Conversely, the number of patients who were added to the liver waiting list for the treatment of NASH increased from 643 in 2008 to 2,100 in 2017 (Organ Procurement and Transplantation Network n.d.). Based on these numbers, it appears that NASH has already surpassed HCV to

become the number one indication for patients to receive liver transplant or will do so in the very near future.

The gold standard for diagnosing NASH is histopathologic evaluation of liver biopsy. The diagnosis of definitive NASH requires the presence of all histologic criteria including steatosis, hepatocellular ballooning, and lobular inflammation. The diagnosis of borderline NASH is given when a patient presents with steatosis and most but not all histologic features of NASH (Chalasani et al. 2018). Several scoring systems have been developed to assess NASH histologic severity, including the NASH Clinical Research Network's NAFLD activity score (NAS), the steatosis, activity, and fibrosis (SAF), and Brunt staging (Bedossa 2014; Kleiner et al. 2005; Brunt et al. 1999). Less invasive methods to assess NASH severity are currently under investigation with some being incorporated into clinical trials. Examples include magnetic resonance imaging (spectroscopy and proton density fat fraction), transient elastography, and serum fibrosis biomarkers (procollagen type III N-terminal protein, tissue inhibitor of metalloproteases 1, hyaluronic acid, cytokeratin-18 fragments) (Vilar-Gomez and Chalasani 2018).

Despite the rising prevalence and burden NASH places on society and the medical system, there is currently no approved therapeutic agent to treat NASH. The current guideline for the management of NASH recommends changes in lifestyles: weight loss, diet, and exercise (Chalasani et al. 2018). Vitamin E and thiazolidinediones may provide benefit to NASH patients, but risks have to be weighed against the potential benefits. Guidelines recommended against using ursodeoxycholic acid (UDCA), metformin, and omega-3 fatty acids for the treatment of NASH, however, can be used to manage concomitant disease states. The guidelines also recommend against the off-label use of obeticholic acid (OCA) until clinical trial data regarding its use for the treatment of NASH become available. The only treatment for patients with advanced fibrotic NASH is liver transplant (Chalasani et al. 2018). With the limited number of organs available for transplant, it is a paramount medical necessity to identify the molecular mechanisms underlying NASH pathogenesis and to develop novel therapies to prevent, mitigate, or reverse NASH development.

2 Introduction: The BA-FXR-FGF19 Pathway

BAs are amphipathic detergents produced in the liver via the hydroxylation of cholesterol (Chiang 2009, 2017). The two predominant pathways responsible for the conversion of cholesterol to BA are the classical (neutral) and alternative (acidic) pathways. In the classical pathway, cholesterol is sequentially oxidized by cytochrome p450 7A1 (CYP7A1) and CYP8B1 to produce cholic acid (CA). The classical pathway accounts for the synthesis of roughly 75% of the total BA pool, and the 7-alpha hydroxylation of cholesterol by CYP7A1 is the rate-limiting step in BA synthesis. The alternative or acidic pathway produces chenodeoxycholic acid (CDCA) by the metabolism of cholesterol by CYP27A1 and CYP7B1. CA and CDCA are conjugated to glycine or taurine by the enzyme bile acid-CoA amino

acid *N*-acyltransferase. CA, CDCA, and their conjugates are considered primary BAs. In the intestine, certain microbial species express the enzyme bile salt hydrolase (BSH) which mediates the deconjugation of BAs. Gut microbes can further metabolize CA and CDCA to the secondary BAs, deoxycholic acid (DCA) and lithocholic acid (LCA) or UDCA, respectively. In mice, UDCA is also a primary BA (Selwyn et al. 2015). Upon reabsorption and reentry to the liver, secondary BAs can be conjugated (Chiang 2009, 2017). The total BA pool therefore consists of numerous species of BAs with unconjugated and conjugated primary and secondary BAs. This will be of later importance as different BA species have different activities (agonist vs. antagonist) and potencies for BA receptors.

BAs undergo significant enterohepatic recirculation with roughly 95% of BAs reabsorbed from the intestine. The majority of BAs are reabsorbed in the ileum into enterocytes by the uptake transporter, apical sodium-dependent BA transporter (ASBT) (Chiang 2009). Once inside enterocytes, BAs can activate the nuclear receptor FXR, and within the nucleus, FXR dimerizes with retinoid X receptor (RXR) to interact with DNA at the FXR response elements (FXRRE) to alter gene transcription (Makishima et al. 1999; Wang et al. 1999; Parks et al. 1999). Activation of FXR in enterocytes leads to the upregulation of fibroblast growth factor 19 (FGF19) in humans and orthologous FGF15 in mice (Inagaki et al. 2005). Though orthologs, FGF15 and FGF19 share only 50% sequence homology (Nishimura et al. 1999; Xie et al. 1999). Both FGF15 and FGF19 are considered endocrine FGFs as they do not bind heparin sulfate and thus can escape extracellular matrix, unlike other families of FGF proteins (Goetz et al. 2007). The structural uniqueness of endocrine FGFs that allow for their systemic circulation also reduces their affinity for fibroblast growth factor receptors (FGFR). Therefore, binding of FGF15 and FGF19 to their predominant receptors FGFR4, and to a lesser extent, FGFR1, requires the obligate co-receptor β-KLOTHO (βKL) (Goetz et al. 2007). Upon induction in the intestine, FGF15/FGF19 travels through portal circulation and activates FGFR4-βKL on hepatocytes (Inagaki et al. 2005; Song et al. 2009; Kurosu et al. 2007). This leads to activation of the extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) signal pathways and subsequently reduces BA synthesis by downregulating the expression of *CYP7A1/Cyp7a1* and *CYP8B1/Cyp8b1* that encode enzymes, CYP7A1 and CYP8B1 (Song et al. 2009; Yu et al. 2005; Kong et al. 2012). FGF15 and FGF19 thereby function as a negative feedback loop shutting down BA synthesis when BA levels are high in the intestinal mucosa. BAs reabsorbed in the intestine activate FXR, transiently increase FGFR4-βKL levels, and prime the liver for subsequent FGF15/FGF19 signaling (Fu et al. 2016). In humans, FGF19 is also expressed at low levels in the liver and is upregulated during cholestasis (Wunsch et al. 2015; Schaap et al. 2009). FXR activation in hepatocytes also suppresses *CYP7A1/Cyp7a1* and *CYP8B1/Cyp8b1* expression by inducing small heterodimer partner (SHP) (Wang et al. 1999; Goodwin et al. 2000; Lu et al. 2000). In hepatocytes, activation of FXR is primarily responsible for promoting BA biliary excretion and does not suppress BA synthesis as strongly as FGF15/FGF19 signaling (Kim et al. 2007).

As described above, there are numerous species of BAs in the body which are produced via enzymatic reactions performed by the liver and gut microbiome. Each BA species has different affinity, efficacy, and potency for each BA receptor. The

strongest endogenous ligand of FXR is CDCA with an EC_{50} of roughly 5 μM (Lew et al. 2004). The most common BA in humans and mice, CA, activates FXR with less efficacy (EC_{50} = ~200 μM) (Parks et al. 1999; Lew et al. 2004; Rizzo et al. 2010). The taurine conjugates of α and β muricholic acid (TαMCA, TβMCA) have been shown to be FXR antagonists (Sayin et al. 2013). MCA is synthesized from precursor CDCA by Cyp2c70, an enzyme expressed in mouse liver but not human liver (Takahashi et al. 2016). MCA is thus a murine-specific BA species. The BA pool composition in mice is predominantly comprised of weak FXR agonist CA (60%) and FXR antagonist MCA (40%). This contrasts to the BA pool in humans comprised of strong FXR agonist CDCA (40%), CA (40%), and DCA (20%). It is currently unclear if the human bile acid pool contains any endogenous FXR antagonists. However, there is growing evidence which supports that UDCA may function as an FXR antagonist (Mueller et al. 2015).

3 Role of FXR in NASH Development in Animal Models

3.1 Systemic FXR

FXR is expressed in many tissues and cell types in the body. Manipulation of body-wide FXR activity either through pharmacologic or genetic means affects the development of each characteristic of NASH: steatosis, inflammation, fibrosis, and metabolic syndrome. This section will broadly describe the effects of systemic FXR activation or deficiency on NASH development. The roles of tissue-specific FXR and the mechanisms by which they influence NASH development will be described in depth in the following sections. FXR agonists used in the animal studies described below include WAY-362450, GW4064, OCA, and fexaramine. For details regarding their structure and properties, please see previous chapters.

Systemic activation of FXR is protective against the development of hepatic steatosis, inflammation, and fibrosis. In mice fed a high-fat diet (HFD), treatment with OCA and GW4064 reduced the accumulation of hepatic triglycerides and free fatty acids and subsequently reduced steatosis severity (Gai et al. 2018; Ma et al. 2013). Similarly, in low-density lipoprotein receptor (*LDLR*) knockout mice fed a Western diet, WAY-362450 reduced hepatic triglyceride and cholesterol levels and attenuated steatosis (Evans et al. 2009). Hepatic inflammation is also reduced by treatment with FXR agonists. In both HFD and methionine and choline-deficient diet (MCD) models, GW4064 and WAY-362450 reduced hepatic inflammation (Ma et al. 2013; Zhang et al. 2009a). Correspondingly, FXR-deficient mice had worsened inflammation induced by MCD (Wu et al. 2014). Activation of whole-body FXR ameliorates hepatic fibrogenesis. OCA, WAY-362450, and BAR704 decreased the severity of fibrosis in mouse HFD, MCD, and carbon tetrachloride (CCl_4) models, respectively (Gai et al. 2018; Ma et al. 2013; Zhang et al. 2009a). Deficiency of FXR worsened fibrosis induced by MCD or knockout of *LDLR* (Wu et al. 2014; Kong et al. 2009).

Body-wide activation of FXR has many beneficial effects on metabolic endpoints. In mice fed a HFD, GW4064 reduced body weights and fat mass. GW4064 also lowered fasting glucose concentrations and improved glucose tolerance. Hepatic gluconeogenesis was also reduced (Ma et al. 2013). Serum lipids are also altered by modulation of whole-body activity of FXR. Activation of FXR by GW4064 and WAY-362450 reduced triglyceride and cholesterol levels in HFD and *LDLR* knockout, Western diet murine models, respectively (Ma et al. 2013; Evans et al. 2009). However, in addition to lowering very low-density lipoprotein (VLDL) and low-density lipoproteins (LDL), WAY-362450 also decreased HDL (Evans et al. 2009). In agreement with the gain-of-function studies, *FXR* knockout mice had increased serum triglyceride, cholesterol, and free fatty acid levels (Ma et al. 2006).

3.2 Hepatic FXR

Multiple cell types in the liver express FXR, including hepatocytes, hepatic stellate cells, endothelial cells, Kupffer cells, and cholangiocytes (Verbeke et al. 2016; Jung et al. 2014; Fiorucci et al. 2004). The role of FXR in inflammatory cells and in regulating inflammatory signaling pathways will be discussed in this section. The activation of FXR locally in the liver affects the development of each characteristic of NASH: steatosis, inflammation, fibrosis, and metabolic syndrome. The effects on each of these characteristics will be described below. For a summary of the effects of FXR activation in specific cell types, please see Fig. 1.

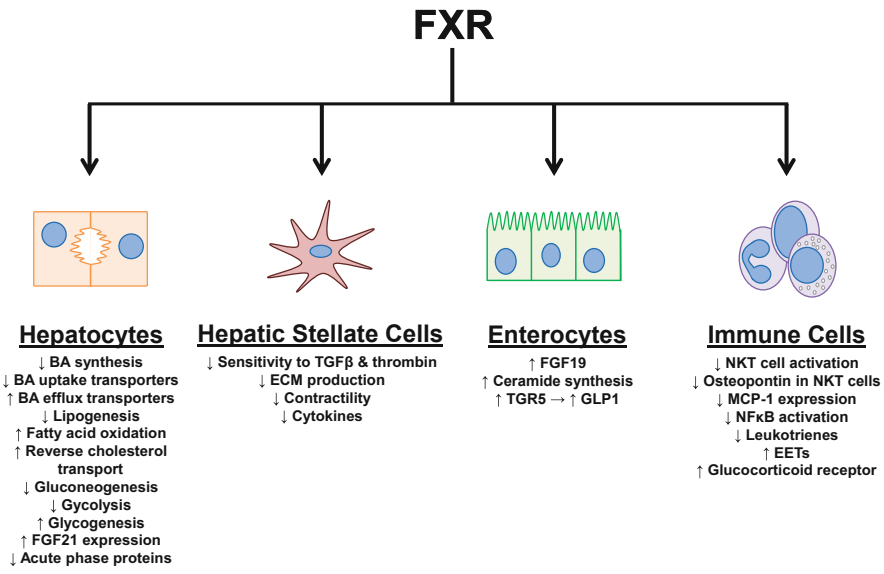

Fig. 1 Summary of the effects of FXR activation in specific cell types

Hepatic FXR activation has been shown to be protective against the development of hepatic steatosis. In a high-cholesterol diet model, hepatic FXR deficiency, but not intestinal FXR deficiency, exacerbated hepatic steatosis (Schmitt et al. 2015). Hepatic FXR activation mitigates hepatic lipid content by decreasing lipogenesis and increasing fatty acid oxidation (Pineda Torra et al. 2003; Watanabe et al. 2004). By inducing SHP, FXR activation decreased sterol regulatory element-binding protein 1c (SREBP1c) expression and consequently decreased the expression of genes involved in lipogenesis (Watanabe et al. 2004). In human hepatocytes, FXR upregulated peroxisome proliferator-activated receptor alpha (PPARα), which subsequently increased fatty acid oxidation (Pineda Torra et al. 2003). It is important to note that the murine PPARα promoter does not have a functional FXRRE. Therefore, in mice PPARα is not an FXR response gene (Pineda Torra et al. 2003). Hepatic FXR also affects lipid homeostasis in the body by enhancing reverse cholesterol transport (Lambert et al. 2003). FXR-deficient mice had reduced expression of scavenger receptor class B type 1 (SRB1), hepatic lipase, cholesterol ester hydrolase, sterol carrier protein, and lecithin-cholesterol acyltransferase and increased expression of apolipoproteins (ApoA-IV, ApoE, and ApoC-III) (Lambert et al. 2003). Hepatic FXR-deficient mice, but not intestinal-deficient FXR mice, had increased serum cholesterol compared to wild type mice when fed a high-cholesterol diet (Schmitt et al. 2015).

In addition to regulating hepatic lipid levels, FXR affects hepatic glucose metabolism. FXR activation decreased gluconeogenesis and glycolysis while increasing glycogenesis. CA-induced FXR activation in mice reduced the hepatic protein levels of enzymes responsible for gluconeogenesis, peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α), phosphoenolpyruvate carboxykinase (PEPCK), and glucose 6-phosphatase (G6Pase). The downregulation of these proteins by CA did not occur in FXR- or SHP-deficient mice indicating that FXR regulates gluconeogenesis in the liver via a SHP-dependent pathway (Ma et al. 2006). Glycogen levels in the liver are increased by FXR activation. In db/db mice, treatment with GW4064 for 5 days increased hepatic glycogen levels by increasing glycogenesis (Zhang et al. 2006). Nonphosphorylated glycogen kinase 3 reduced glycogen synthase activity. However, this effect was reduced by phosphorylation (Plyte et al. 1992). Levels of phosphorylated glycogen kinase 3 were increased in the GW4064 treated mice. GW4064 treatment also increased the phosphorylation of insulin receptors 1 and 2. Therefore, FXR may also increase hepatic glycogen levels by enhancing insulin sensitivity (Zhang et al. 2006). In agreement with the previous gain-of-function study, FXR-deficient mice had reduced levels of hepatic glycogen (Cariou et al. 2005). FXR activity can also increase hepatic glycogen levels by suppressing glycolysis. Pyruvate dehydrogenase complex (PDC) is an important metabolic switch that regulates the oxidation of glucose for fatty acid synthesis. Pyruvate dehydrogenase kinase 4 (PDK4) inhibits PDC and reduces glycolysis (Sun et al. 2015). In vitro treatment of human hepatocytes and in vivo treatment of mice with FXR agonist GW4064 increased expression of PDK4 and thus decreased glycolysis (Savkur et al. 2005).

The metabolic effects of FXR in the liver may also be mediated by fibroblast growth factor 21 (FGF21). The promotor of *FGF21* has a functional FXRRE, and the

expression of *FGF21* in the liver has been shown to be regulated by FXR. However, FGF21 is predominantly regulated by PPARα (Badman et al. 2007; Cyphert et al. 2012). In vivo treatment of mice and in vitro treatment of human hepatocytes with CDCA increase FGF21 expression and secretion (Cyphert et al. 2012). Numerous studies have demonstrated the effects of FGF21 on NASH and metabolic endpoints, which has been the subject of many review articles (Zhang et al. 2015; Staiger et al. 2017; Potthoff 2017; Markan and Potthoff 2016; Guan et al. 2016; Nies et al. 2015). In brief, FGF21 increases browning of adipose tissue, energy expenditure, insulin production, glucose uptake by white adipose tissue, gluconeogenesis, ketogenesis, and lipolysis. In NASH models, FGF21 is protective against hepatic steatosis, inflammation, fibrosis and metabolic syndrome (Lee et al. 2016; Liu et al. 2016; Fisher et al. 2014). To our knowledge, in studies using FXR agonists, the extent to which the FXR-FGF21 axis affects NASH or metabolic disease development has not been shown.

FXR activation is anti-inflammatory and affects both innate and adaptive immune responses. Innate immune responses shown to be affected by FXR include the acute phase response and natural killer T-cell (NKT) activation. The acute phase response is a systemic reaction to local or systemic acute infection, illness, or injury (Cray et al. 2009). During the acute phase response, the expression of acute phase proteins, which are predominantly produced in hepatocytes, is markedly altered (normally increased). In humans, the major acute phase protein is C-reactive protein (CRP), whereas in mice the major acute phase proteins are serum amyloid P component (SAP) and serum amyloid A3 (SAA3) (Cray et al. 2009). FXR activation has been shown to reduce the expression of CRP, SAP, and SAA3. In Hep3B cells, FXR agonism with GW4064 and WAY-362450 mitigated the induction of CRP by interleukin-6 (Zhang et al. 2009b). Treatment of mice with WAY-362450 reduced LPS-stimulated induction of SAP and SAA3, whereas knockout of FXR increased the induction of SAP and SAA3 (Zhang et al. 2009b). In contrast, FXR activation, at least in mice, has been shown to induce the expression of a cohort of genes involved in acute phase response (Armstrong et al. 2017; Porez et al. 2013). The exact role of FXR in regulating acute phase response needs further investigation. FXR may also affect the innate immune system by regulating the activation of liver NKT cells. NKT cells have been shown to express both FXR and SHP. In NKT cells, activation of FXR induces SHP, which prevents the binding of c-Jun to the osteopontin promoter (Mencarelli et al. 2009). Osteopontin has many effects on immune cells including chemotaxis, cellular adhesion, and cell survival (Wang and Denhardt 2008). In the Con A model of acute hepatitis, OCA treatment reduced the number of FasL-positive NKT cells indicating the FXR may mediate NKT-cell activation (Mencarelli et al. 2009).

The adaptive immune system is regulated by FXR by several mechanisms, including directly altering inflammatory mediator expression, antagonism of the nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) pathway, and enhancing glucocorticoid signaling. Monocyte chemoattractant protein 1 (MCP-1) is a chemokine that regulates monocyte and macrophage migration and infiltration (Deshmane et al. 2009). An FXRRE is present in the promoter of MCP-1. Activation of FXR by CDCA in macrophage cell lines, ANA-1 and RAW264.7, reduced both mRNA and protein levels of MCP-1 (Li et al. 2015). In primary isolated Kupffer cells,

OCA mitigated the upregulation of MCP-1 by both lipopolysaccharide (LPS) and tumor necrosis factor alpha (TNFα) (Verbeke et al. 2016). In the MCD model of NASH, treatment of mice with FXR agonist WAY-362450 decreased MCP-1 expression in the liver and reduced inflammatory infiltrate (Zhang et al. 2009a).

Another mechanism by which FXR is anti-inflammatory is through the inhibition of the NFκB signaling pathway. Posttranslational modification of FXR can occur at residue K277. This lysine can either be acetylated or SUMOylated. When SUMOylated, FXR can tether to NFκB subunit p65 and prevent the recruitment of p65 to the promoter of its inflammatory response genes. FXR activation increased the amount of SUMOylated FXR and consequently reduced NFκB signaling (Kim et al. 2015). Treatment of mice with FXR agonists reduced the induction of inflammatory mediators by LPS challenge (Wang et al. 2008). Similarly, preventing FXR activity or SUMOylation increases inflammatory mediator expression. When challenged with LPS, FXR-deficient mice have higher induction of NFκB response genes (Wang et al. 2008). FXR may also reduce NFκB activation by increasing levels of nuclear factor of kappa light polypeptide gene enhancer in B-cell inhibitor alpha (IκBα), the chaperone protein which prevents the translocation of p65 to the nucleus. In the thioacetamide model of cirrhosis, mice treated with OCA had increased hepatic protein levels of IκBα (Verbeke et al. 2016). Lastly, FXR has recently been shown to decrease NFκB pathway activation by increasing the production of anti-inflammatory arachidonic acid-derived epoxyeicosatrienoic acids (EETs) and reducing production of inflammatory leukotrienes. During NASH development in humans, the cytochrome p450s which produce EETs are reduced, and expression levels are inversely correlated to NAS score (Gai et al. 2018). EETs have been previously shown to reduce NFκB activation (Dai et al. 2015). In mice fed free fatty acids, OCA increased the expression of cytochrome p450s that synthesize EETs and reduced hepatic inflammation (Gai et al. 2018).

In addition to modulating the activity of the NFκB pathway, FXR regulates glucocorticoid signaling. An FXRRE was identified in the distal portion of the murine and human glucocorticoid receptor promoter (Renga et al. 2012, 2013). As evidenced by chromatin immunoprecipitation and luciferase assay, FXR was recruited to this FXRRE but did not directly alter gene transcription. Instead, the FXRRE functions as an enhancer element, and FXR recruitment to this FXRRE mediates chromatin head-to-tail looping, thereby increasing transcriptional efficiency (Renga et al. 2013). Primary monocytes from wild type and FXR-deficient mice were treated with LPS and dexamethasone. Monocytes from FXR-deficient mice were less responsive to the anti-inflammatory effects of dexamethasone and had elevated inductions of *Il-1β*, *Tnfα*, and *interferon-γ* (Renga et al. 2013).

Hepatic stellate cells (HSCs) also express FXR in the liver albeit primary isolated rat HSCs express low levels of FXR compared to liver tissue homogenate (Fickert et al. 2009). The rat HSC cell line HSC-T6 and human HSC cell line LX-2 also express FXR (Fiorucci et al. 2005a). Activation of FXR in HSCs affects numerous signaling pathways, which, together, function to reduce hepatic fibrosis. The expression of SHP is induced in HSCs by activation of FXR (Fiorucci et al. 2004; Carino et al. 2018). In HSCs, SHP binds to SMAD3 and JunD (Fiorucci et al. 2004; Carino

et al. 2018). By binding to SMAD3, SHP prevents SMAD3 from interacting with the transforming growth factor beta (*TGFβ*) promoter and reduces HSC responsiveness to TGFβ (Carino et al. 2018). Induction of *collagen 1α1* (Col1α1) by TGFβ in HSC-T6 cells was reduced by CDCA (Fiorucci et al. 2004). In LX-2 cells, OCA treatment reduced TGFβ inductions of *COL1α1*, *alpha smooth muscle actin* (*αSMA*), *matrix metalloprotease 2* (*MMP2*), *transforming growth factor beta receptor 2* (*TGFβR2*), *TGFβ*, and *endothelin-1* (*ET-1*) (Carino et al. 2018). Through binding to JunD, SHP reduced the binding of activator protein-1 (AP-1) to DNA, thereby preventing HSC activation induced by thrombin (Fiorucci et al. 2004). OCA treatment of primary rat HSCs and HSC-T6 cells attenuated the induction of *tissue inhibitor of metalloproteases 1* (*Timp1*) by thrombin and increased MMP2 activity in a SHP-dependent manner (Fiorucci et al. 2005a).

FXR activation also induces the expression of peroxisome proliferator-activated receptor gamma (PPARγ) in HSCs (Fiorucci et al. 2005b). The promoter of PPARγ has been shown to contain a functional FXRRE by a luciferase assay (Renga et al. 2011). By inducing PPARγ, FXR activation in HSCs reduced the expression of inflammatory cytokines (Renga et al. 2011). PPARγ is also a negative regulator of collagen expression. During HSC transdifferentiation to an activated phenotype, PPARγ expression is markedly reduced, and expression of collagen increases. Treatment of primary rat HSCs with OCA mitigated the downregulation of *PPARγ* by random transdifferentiation in culture and reduced collagen expression (Fiorucci et al. 2005b). Primary HSCs were isolated from OCA-treated rats that underwent either the porcine serum, bile duct ligation, or CCl_4 liver fibrosis models. HSCs from the OCA-treated animals had higher expression of PPARγ (Fiorucci et al. 2005b). FXR also decreases extracellular matrix production by increasing the expression of miRNA-29a in HSCs (Li et al. 2011). An FXRRE was identified in the miRNA-29a promoter. The expression of extracellular matrix proteins, collagen, elastin, and fibrillin was reduced by miRNA-29a (Li et al. 2011).

HSC contractility is regulated by FXR. The expression of dimethylarginine dimethylaminohydrolase 2 (DDAH2) is upregulated in HSCs by FXR activation (Verbeke et al. 2014). This leads to increased activity of endothelial nitric oxide synthase (eNOS) as DDAH2 degrades asymmetric dimethylarginine and monomethyl-L-arginine, inhibitors of NOS (Verbeke et al. 2014; Vallance et al. 1992). FXR also decreases HSC contractility by decreasing the expression of ET-1 (Xu et al. 2016; Li et al. 2010). Reductions in ET-1 reduces Rho-associated protein kinase pathway activation and reduces the phosphorylation of myosin light chain. FXR activation also reduces phosphorylation of myosin light chain by reducing myosin light chain kinase levels (Xu et al. 2016). In summary, FXR activation in HSCs reduces extracellular matrix production while increasing extracellular matrix degradation, reduces HSC responsiveness to pro-fibrotic mediators, reduces inflammatory mediator expression, and reduces HSC contractility.

3.3 Intestinal FXR

The role of intestinal FXR during NASH and metabolic disease development is currently unclear. Both inhibition and activation of FXR in the intestine has been shown to have beneficial effects in animal models. In this section we will review the data from studies using both intestinal-specific FXR antagonists and agonists.

The beneficial effects of intestinal FXR antagonism on NASH and metabolic diseases are mediated through a microbiome-intestine-liver ceramide axis (Jiang et al. 2015a, b; Xie et al. 2017). In the intestine, FXR has been shown to upregulate the genes involved in ceramide synthesis (Jiang et al. 2015a, b). Ceramide synthesized in the intestine entered circulation, increased SREBP1c activity in the liver, and subsequently increased lipogenic gene expression (Jiang et al. 2015a). Mice fed a HFD were treated with the BA-based FXR antagonist, glycine-conjugated MCA (Gly-MCA) (Jiang et al. 2015b). Gly-MCA reduced hepatic triglyceride accumulation. Gly-MCA also reduced total body weight and fasting insulin levels, improved insulin sensitivity, and led to the browning of adipose tissue. The beneficial effects of Gly-MCA were prevented by co-treatment with ceramide and the FXR agonist GW4064 (Jiang et al. 2015b). Additionally, treatment of mice with tempol or antibiotics modified the microbiome and increased levels of TβMCA, an FXR antagonist. By increasing TβMCA and inhibiting intestinal FXR, tempol and antibiotic treatment reduced HFD-induced hepatic steatosis (Jiang et al. 2015a). In a similar study, mice fed a HFD were treated with caffeic acid phenethyl ester (CAPE), a BSH inhibitor (Xie et al. 2017). CAPE treatment increased ileal levels of TβMCA, thereby reducing intestinal FXR activity and ceramide synthesis. CAPE-treated mice had reduced body weights, reduced fasting glucose and insulin levels, and improved glucose tolerance. By reducing ceramide levels, CAPE treatment also reduced hepatic endoplasmic reticulum stress and hepatic gluconeogenesis (Xie et al. 2017). Intestine-specific knockout of FXR reduced HFD-induced hepatic triglyceride accumulation and steatosis development (Li et al. 2013).

Reports have also been published demonstrating the benefits of intestinal FXR agonism in animal models. Due to poor systemic bioavailability, fexaramine is an intestinal-specific FXR agonist when administered orally (Fang et al. 2015). In HFD models, mice treated with fexaramine had reduced body weight and body fat mass, increased browning of adipose tissue, and increased energy expenditure (Fang et al. 2015; Pathak et al. 2018). Fexaramine treatment reduced expression of genes involved in lipogenesis, triglyceride levels, and steatosis in the liver (Fang et al. 2015; Pathak et al. 2018). Glycemic endpoints were also improved by fexaramine, including reduced fasting serum insulin and leptin levels, increased serum glucagon-like peptide-1 (GLP1) levels, improved insulin sensitivity, and reduced hepatic gluconeogenesis (Pathak et al. 2018). Fexaramine increased intestinal barrier function and decreased circulating levels of inflammatory mediators (Fang et al. 2015).

The effects of intestinal FXR activation described above are mediated through multiple pathways: FXR-Takeda G-protein receptor 5 (TGR5) cross talk and induction of FGF15/FGF19. *TGR5* has been shown to be an FXR response gene. The promoter of *TGR5* has a functional FXRRE, and FXR activation increases TGR5

mRNA transcript and protein levels (Pathak et al. 2017, 2018). Not only does intestinal FXR activation increase TGR5 levels but also increases TGR5 ligands. Fexaramine shifts the BA pool composition to contain markedly higher levels of TLCA and LCA, both strong agonists of TGR5 (Kawamata et al. 2003; Maruyama et al. 2002). TGR5 activation in the intestine increases serum GLP1 levels. Therefore, the increases in GLP1 levels in fexaramine-treated mice are the consequence of enhanced TGR5 signaling. Knockout of either *Fxr* or *Tgr5* prevented fexaramine from inducing serum GLP1 concentration and browning of adipose tissue (Pathak et al. 2018). The effects of fexaramine can also be resultant of induction of FGF15. In the intestine, *Fgf15* is an FXR target gene. Fexaramine treatment increased intestinal *Fgf15* expression and circulating FGF15 protein levels (Fang et al. 2015; Pathak et al. 2018). FGF15 and FGF19 have many beneficial effects on NASH and metabolic diseases, which will be described in depth in the following section.

3.4 FGF19

Many of the effects stimulated by activation of intestinal FXR are mediated through the regulation of FGF19. As previously described, FXR activation in the intestine leads to the upregulation of FGF19 (Inagaki et al. 2005). Unlike most FGFs, FGF19 does not bind heparin sulfate and therefore can circulate systemically (Goetz et al. 2007). The tissue-specific activities of FGF19 are determined by the distribution of FGFR1, FGFR4, and co-receptor βKL throughout the body (Kurosu et al. 2007). FGF19 has been shown to regulate the functions of numerous organs paramount to the development of NASH and metabolic diseases including the liver, adipose, muscle, and brain. The effects of FGF19 on each of these organs and subsequent effects on NASH and metabolic diseases will be discussed below. For a summary of the effects of FGF19 signaling in specific cell types, please see Fig. 2.

In the liver, FGF15 and FGF19 prevent the development of the major characteristics of NASH: steatosis, inflammation, fibrosis, and metabolic syndrome. FGF19 gain-of-function studies, either from transgenic overexpression or treatment with recombinant or modified FGF19 protein, have shown that FGF19 is protective against triglyceride and cholesterol accumulation in the liver and thereby decreases steatosis (Tomlinson et al. 2002; Zhou et al. 2017a; Alvarez-Sola et al. 2017). In agreement, a loss-of-function study found that *Fgf15* knockout mice fed a HFD have worsened steatosis (Alvarez-Sola et al. 2017). A second study which fed a HFD to *Fgf15* knockout mice did not find worsened steatosis severity but did find altered expression of lipid homeostatic genes (Schumacher et al. 2017). FGF15 and FGF19 reduce steatosis by negatively regulating genes involved in lipid synthesis (*Fas*, *Acly*, *Fatp4*, *Elovl6*, *Scd1*, *Mogat1*, *Dgat2*, *Scd1*) and lipid uptake (*Cd36*) (Tomlinson et al. 2002; Zhou et al. 2017a; Alvarez-Sola et al. 2017; Schumacher et al. 2017). FGF19 has also been shown to reduce steatosis development through altering the composition of the BA pool to contain increased TβMCA. The increased TβMCA levels antagonize intestinal FXR activity and decreased intestinal ceramide synthesis. As previously described (see Sect. 3.3), reduced intestinal ceramide

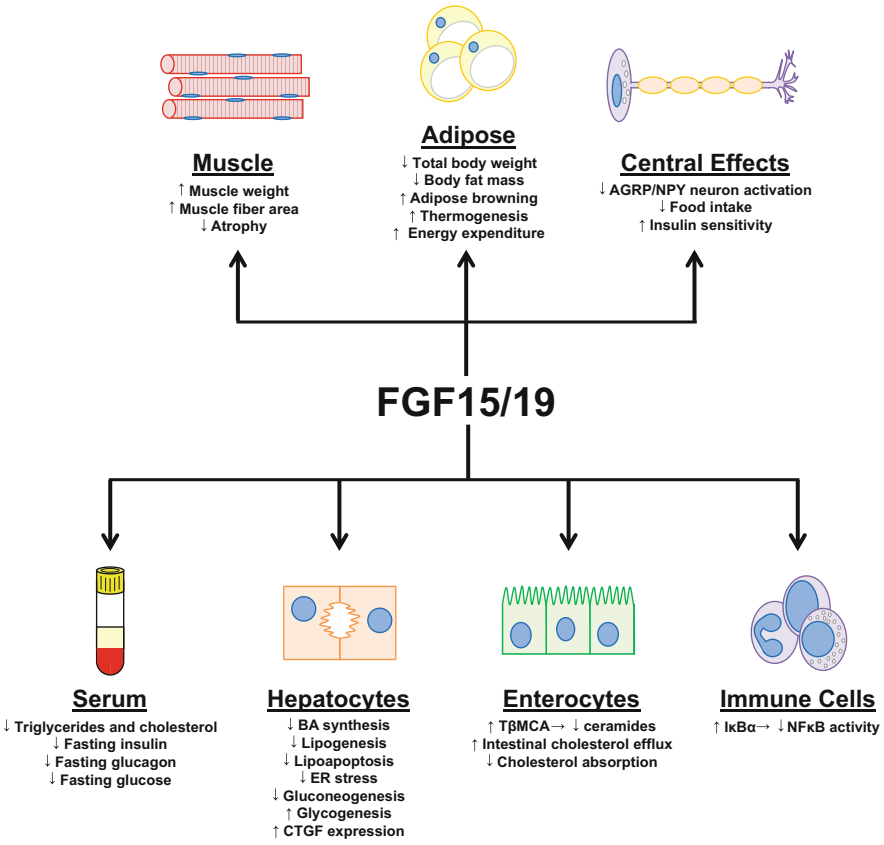

Fig. 2 Summary of the effects of FGF19 signaling in specific cell types

production decreases SREBP1c activation in the liver and subsequently mitigates steatosis (Zhou et al. 2017a). In addition to reducing lipid accumulation, FGF19 protects hepatocytes against lipoapoptosis and reduces endoplasmic reticulum stress (Zhou et al. 2017a; Alvarez-Sola et al. 2017). By altering the BA pool, FGF19 also reduces enterocyte cholesterol absorption, increases transintestinal cholesterol efflux, and increases fecal sterol content (de Boer et al. 2017).

FGF15 and FGF19 reduce the development of hepatic inflammation. In a high-fat, high-fructose, high-cholesterol diet mouse model, overexpression of FGF19 or modified FGF19 protein (M70, NGM282) reduced hepatic inflammation severity observed histologically and reduced expression of inflammatory mediators (Zhou et al. 2017a). Though not significant, FGF15-deficient mice fed a HFD had trends for worsened inflammation. One mechanism by which FGF19 may mitigate hepatic inflammation is via altering NFκB activity. FGFR4 activation by FGF19 has been shown to reduce NFκB signaling. Activated FGFR4 interacted with inhibitor of

nuclear factor kappa-B kinase subunit beta (IKKβ) and decreased IKKβ-mediated phosphorylation of IκBα (Drafahl et al. 2010).

The effect of FGF15 and FGF19 on the development of hepatic fibrosis is currently unclear. In the aforementioned high-fat, high-fructose, high-cholesterol mouse model, FGF19 and M70 overexpression markedly reduced the development of hepatic fibrosis (Zhou et al. 2017a). However, in both HFD-induced NASH model and CCl_4 hepatic fibrosis model, FGF15 deficiency was protective against hepatic fibrosis (Schumacher et al. 2017; Uriarte et al. 2015). In a study using the CCl_4 model, connective tissue growth factor (*CTGF*) was shown to be a FGF15 and FGF19 target gene in hepatocytes. Knockout of *Fgf15* reduced hepatocyte-derived CTGF and ameliorated CCl_4-induced fibrosis. *Fgf15* knockout also increases total BA pool size and therefore may increase FXR activity in HSCs, which as described previously reduces HSC activation, responsiveness to TGFβ, extracellular matrix production, and contractility. In the FGF19 gain-of-function study, it is possible the reduced fibrosis was resultant of mitigated hepatic steatosis and inflammation, thus reducing the intensity of HSC activating signals.

FGF19 has beneficial effects on the metabolic syndrome: mitigating dyslipidemia, improving glucose homeostasis, reducing total body weights, and reducing body fat mass. Overexpression of FGF19 reduces serum triglyceride and total cholesterol levels (Tomlinson et al. 2002). In mice fed a diet high in fat, fructose, and cholesterol, FGF19 overexpression reduced triglyceride, total cholesterol, and LDL levels (Zhou et al. 2017a). Conversely, FGF15 deficiency in mice increases serum triglyceride levels induced by HFD (Schumacher et al. 2017). Fasting serum glucose and insulin levels are decreased by FGF15 and FGF19. Mice overexpressing FGF19 had reduced fasting serum insulin, glucagon, and glucose levels (Tomlinson et al. 2002; Zhou et al. 2017a). These mice also had improved responses during insulin and glucose tolerance tests (Tomlinson et al. 2002; Zhou et al. 2017a). Homeostatic model assessment of β-cell function and insulin resistance (HOMA-IR), an indicator of insulin resistance, was reduced in transgenic mice (Zhou et al. 2017a). The reverse was observed in FGF15-deficient mice which had increased fasting glucose levels and worsened glucose tolerance (Schumacher et al. 2017; Kir et al. 2011). FGF19 also affects glucose homeostasis in the body by regulating hepatic gluconeogenesis and glycogenesis. In the liver, FGF19 activation of FGFR4-βKL causes the dephosphorylation and inactivation of the cAMP response element-binding protein (CREB) and consequently leads to the downregulation of *Pgc1α* expression. The lower levels of PGC1α decreases the expression of *Pepck* and *G6Pase*, genes involved in gluconeogenesis. Knockout of *Fgf15* increased *Pgc1α*, *Pepck*, and *G6Pase* expression (Potthoff et al. 2011). Liver glycogenesis is also regulated by FGF15 and FGF19. Liver homogenates from FGF19-treated mice had increased glycogen synthase activity and increased levels of glycogen, whereas FGF15-deficient mice had reduced glycogenesis post glucose challenge (Kir et al. 2011). The mechanism by which FGF19 increases hepatic glycogenesis is shown to be dependent upon ERK signaling and independent of insulin signaling (Kir et al. 2011).

FGF19 also affects NASH and metabolic disease development by its effects peripherally on adipose and muscle tissue. Adipose tissue does not express FGFR4,

and regulation of adipose tissue by FGF19 is mediated by FGFR1-βKL (Kurosu et al. 2007; Tomlinson et al. 2002). Treatment of mice with FGF19 and transgenic overexpression of FGF19 reduces body fat mass and total body weight (Tomlinson et al. 2002; Zhou et al. 2017a; Benoit et al. 2017). When fed a HFD, FGF19 transgenic mice resisted body weight gain and expansion of retroperitoneal and epididymal white adipose tissue (Tomlinson et al. 2002). Correspondingly, knockout of *Fgf15* increased fat mass and total body weight during high-fat feeding (Alvarez-Sola et al. 2017). FGF19 transgenic mice have shown to have increased brown adipose tissue, thermogenesis, and energy expenditure (Tomlinson et al. 2002; Fu et al. 2004).

In addition to its effects on adipose tissue, FGF19 also regulates muscle tissue. Treatment of mice with FGF19 increased soleus, tibialis anterior, and gastrocnemius muscle weights in a βKL-dependent manner; the number of muscle fibers was not altered by FGF19, but instead fiber area was increased (Benoit et al. 2017). Concordantly, human myotubes treated in vitro with FGF19 have increased area. FGF19 also protects against dexamethasone, obesity, and age-induced muscle atrophy. Reductions in atrophy by FGF19 further manifested as improvements in grip strength, an indicator of muscle strength (Benoit et al. 2017).

FGF19 not only regulates body weight and glucose homeostasis peripherally but also acts centrally in the brain. A study using radiolabeled iodinated FGF19 examined its pharmacokinetic properties after intravenous injection. After 10 min, radiolabeled intact ^{125}I-FGF19 was present in the brain though at low levels. Brain perfusion indicates that FGF19 does cross the blood-brain barrier (BBB), but to a limited extent (Hsuchou et al. 2013). It is important to note that FGF15 and FGF19 are expressed in the developing fetal brain. However, it is not expressed in the adult brain (Nishimura et al. 1999; Gimeno et al. 2003; Fon Tacer et al. 2010). It is therefore likely that FGF15 and FGF19 exert their central effects not by crossing the BBB but instead by interacting with neurons that have projections that traverse the BBB, one such neuron type being the agouti-related peptide (AGRP)/neuropeptide Y (NPY) neurons (Faouzi et al. 2007). In the arcuate nucleus of the hypothalamus, AGRP/NPY neurons express FGFR1, FGFR2, and FGFR3 but not FGFR4 (Liu et al. 2018). As shown by immunofluorescence, intraperitoneal injection of FGF19 in mice increased phosphorylation of the FGFR secondary messenger ERK in NPY neurons in the hypothalamic arcuate nucleus (Marcelin et al. 2014). FGF19 signaling decreases the activation of AGRP/NPY neurons (Marcelin et al. 2014). Expression of c-Fos is a marker of neuron activation (Bullitt 1990). HFD-fed mice and ob/ob mice have increased NPY/c-Fos-co-positive cells in the hypothalamus. FGF19 given by intracerebral-ventricular injection (*icv*) decreased the number of NPY/c-Fos-positive cells in HFD mice (Marcelin et al. 2014). The effects of *icv* FGF19 on metabolic disease development have been studied in both ob/ob and HFD mouse models (Fu et al. 2004; Marcelin et al. 2014; Ryan et al. 2013). In these studies, *icv* FGF19 reduced food intake and body weight gain, improved glucose and insulin tolerance, and decreased fasting insulin levels. Inhibition of FGFR in the brain via *icv* injection of FGFR inhibitor PD173074 had the opposite effects: increased food intake, total body weight, and worsened insulin tolerance (Ryan et al. 2013). Taurocholic acid (TCA) feeding was shown to increase FGF15 levels and increase

glucose tolerance. Tissue-specific knockout of *Fgfr1* in AGRP neurons prevented the improvement of glucose tolerance by TCA. These findings indicate the beneficial central effects of FGF15 and FGF19 on glucose homeostasis are likely mediated by FGF19 activation of FGFR1 centrally (Liu et al. 2018).

A bi-specific activating antibody (bFKB1) targeting FGFR1-βKL has been designed and tested in mice and cynomolgus monkeys (Chen et al. 2017; Kolumam et al. 2015). As the effects of FGF19 on adipose tissue and brain are mediated by FGFR1-βKL, the effects of bFKB1 should mirror the extrahepatic effects of FGF19 but not the hepatic FGFR4-mediated effects. As expected, bFKB1 decreased body weight while increasing browning of adipose tissue, thermogenesis, and energy expenditure. Treatment with bFKB1 also reduces blood glucose and insulin levels, improved glucose tolerance, reduced hepatic triglycerides, and reduced serum lipids. Interestingly, the effects of bFKB1 on brown fat thermogenesis were still present in adipocyte-specific FGFR1-deficient mice and in uncoupling protein 1 (UCP1)-deficient mice indicating the effects on thermogenesis may be mediated indirectly (Chen et al. 2017). In both mice and cynomolgus monkeys, bFKB1 treatment led to inductions of high molecular weight adiponectin. Changes in body weight and energy expenditure are also independent of effects on adiponectin; body weight and energy expenditure changes were also present in adiponectin deficient mice (Kolumam et al. 2015). It is possible that the effects of bFKB1 are mediated centrally. Of importance, treatment with bFKB1 did not induce phosphorylation of ERK in the liver (Kolumam et al. 2015). This is promising as one of the potential liabilities of FXR agonists and FGF19 analog therapeutics is FGF19-FGFR4-driven hepatocellular carcinoma (See later Sect. 5).

4 Progress of FXR Agonists in Human Clinical Trials

4.1 FXR Agonists

The development of FXR agonists for the treatment of NASH is currently a hotbed of research. Several compounds currently in human clinical trials and one compound, obeticholic acid (OCA), have already been approved for the treatment of another liver disease. These agonists have both steroidal and nonsteroidal pharmacophores and activate FXR systemically. Current progress of these compounds in clinical trials is described below and summarized in Table 1.

OCA received accelerated approval for the treatment of primary biliary cirrhosis (PBC) in 2016. The accelerated approval was based upon reductions in alkaline phosphatase (ALP) in PBC patients and was given with the condition that improvements in survival or disease outcomes be established (Ocaliva (obeticholic acid) 2018). To ascertain this information, the FDA required three additional studies: (1) a pharmacokinetic, safety, and efficacy study in PBC patients with Child-Pugh classes B and C, (2) a safety and efficacy study of OCA for the monotherapy of PBC in patients intolerant or unresponsive to UDCA, and (3) a study in PBC patients demonstrating that observed decreases in ALP are associated with changes in

Table 1 List of completed and ongoing clinical trials investigating the use of FXR agonists and FGF19 analogs for the treatment of NASH

Mechanism	Compound	Phase	Study title	Start date	End date	NCT ID#
FXR agonist	OCA	III	Randomized global Phase III study to evaluate the impact on NASH with fibrosis of obeticholic acid treatment (REGENERATE)	September 2015	October 2022	NCT02548351
		III	Study evaluating the efficacy and safety of obeticholic acid in subjects with compensated cirrhosis due to nonalcoholic steatohepatitis (REVERSE)	August 2017	July 2021	NCT03439254
		II	The farnesoid X receptor (FXR) ligand obeticholic acid in NASH treatment trial (FLINT)	March 2011	September 2014	NCT01265498
		II	An exploratory study of INT-747 in patients with diabetes mellitus and presumed NAFLD	July 2007	February 2009	NCT00501592
		I	Obeticholic acid in morbidly obese patients and healthy volunteers (OCAPUSH)	August 2015	October 2019	NCT02532335
		I	Hepatic impairment trial of obeticholic acid	June 2013	October 2013	NCT01904539
		I	Effect of food on pharmacokinetics of obeticholic acid (OCA)	August 2013	November 2013	NCT01914562
		I	Single-dose and multiple-dose trial to assess pharmacokinetics of obeticholic acid (OCA)	October 2013	November 2013	NCT01933503
	Tropifexor (LJN452)	II	Safety, tolerability, and efficacy of a combination treatment of tropifexor (LJN452) and cenicriviroc (CVC) in adult patients with nonalcoholic steatohepatitis (NASH) and liver fibrosis (TANDEM)	August 2018	June 2020	NCT03517540
		II	Study of safety and efficacy of tropifexor (LJN452) in patients with nonalcoholic steatohepatitis (NASH) (FLIGHT-FXR)	August 2016	September 2019	NCT02855164
	EDP-305	II	A study to assess the safety, tolerability, pharmacokinetics, and efficacy of EDP-305 in subjects with nonalcoholic steatohepatitis	April 2018	April 2019	NCT03421431
		I	Drug-drug interaction study between EDP-305, itraconazole, and rifampin in healthy volunteers	July 2017	September 2017	NCT03213145

		I	A study of EDP-305 in subjects with mild and moderate hepatic impairment compared with normal healthy volunteers	June 2017	September 2017	NCT03207425
		I	Drug-drug interaction study between EDP-305, midazolam, caffeine, and rosuvastatin in healthy volunteers	May 2017	June 2017	NCT03187496
		I	A study of EDP 305 in healthy subjects and subjects with presumptive NAFLD	September 2016	June 2017	NCT02918929
	GS-9674	II	Safety and efficacy of selonsertib, GS-0976, GS-9674, and combinations in participants with bridging fibrosis or compensated cirrhosis due to nonalcoholic steatohepatitis (NASH) (ATLAS)	March 2018	April 2020	NCT03449446
		II	Safety, tolerability, and efficacy of selonsertib, GS-0976, and GS-9674 in adults with nonalcoholic steatohepatitis (NASH)	July 2016	July 2019	NCT02781584
		II	Evaluating the safety, tolerability, and efficacy of GS-9674 in participants with nonalcoholic steatohepatitis (NASH)	October 2016	January 2018	NCT02854605
		I	Pharmacokinetics and pharmacodynamics of GS-9674 in adults with normal and impaired hepatic function	July 2016	December 2018	NCT02808312
		I	Study in healthy volunteers to evaluate the safety, tolerability, pharmacokinetics, and pharmacodynamics of GS-9674 and the effect of food on GS-9674 pharmacokinetics and pharmacodynamics	January 2016	July 2016	NCT02654002
	Nidufexor (LMB763)	II	Safety, tolerability, pharmacokinetics, and efficacy of LMB763 in patients with NASH	October 2016	March 2019	NCT02913105
	Turofexorate (FXR450)	I	Study evaluating the safety of FXR450 in healthy subjects	October 2007	February 2008	NCT00499629

(continued)

Table 1 (continued)

Mechanism	Compound	Phase	Study title	Start date	End date	NCT ID#
	EYP001	I	Study evaluating safety, tolerability, and pharmacokinetics of EYP001a in healthy male subjects	August 2016	March 2017	NCT03110276
FGF19 analog	NGM282	II	Study of NGM282 in patients with nonalcoholic steatohepatitis (NASH)	May 2015	September 2019	NCT02443116
		I/II	Study of NGM282 in subjects with functional constipation and healthy individuals	December 2015	January 2017	NCT02649062
		I	SAD and MAD study of NGM282 in healthy adult participants	January 2013	July 2013	NCT01776528
FGFR1-βKL activating antibody	NGM313	I	Study of NGM313 in obese participants	September 2017	December 2018	NCT03298464
		I	Study of NGM313 in healthy adult participants	February 2016	April 2017	NCT02708576

clinical progression to cirrhosis, transplant, decompensation, or death (FDA approval letter – Ocaliva 2016). These trials are to be completed by the end of 2022 (FDA approval letter – Ocaliva 2016). For the treatment of NASH, OCA has completed two Phase II trials with Phase III trials underway (REGENERATE and REVERSE) (Mudaliar et al. 2013; Neuschwander-Tetri et al. 2015; Intercept Pharmaceuticals 2018a, b).

In the Phase II study, the safety and efficacy of OCA was investigated in patients with NAFLD and type 2 diabetes mellitus (Mudaliar et al. 2013). Sixty-four patients were randomized to placebo ($n = 23$), 25 mg of OCA ($n = 20$), and 50 mg of OCA ($n = 21$). The primary endpoint was changes in insulin sensitivity determined by glucose infusion rate during two-step euglycemic clamp procedure. Insulin sensitivity was improved in patients in the low-dose group and trended for improvement in the high-dose group. Many additional secondary endpoints were also measured including changes in body weight, serum biomarkers of liver injury, serum biomarkers of BA homeostasis, and fibrosis biomarkers. As expected, OCA increased serum FGF19 levels, suppressed BA synthesis indicated by decreased serum C4 levels (intermediate of BA synthesis used as biomarker of BA synthesis), and reduced serum BA concentrations. OCA had many beneficial effects in patients including reduced body weights, serum triglyceride levels, serum alanine aminotransferase (ALT) and γ-glutamyltransferase (GGT) activities, and reduction in fibrosis biomarkers. However, of potential concern, OCA increased levels of serum LDL while lowering HDL. Serum ALP levels were also increased in OCA-treated patients (Mudaliar et al. 2013).

The "FXR ligand obeticholic acid for non-cirrhotic, non-alcoholic steatohepatitis trial" (FLINT) was a multicenter, randomized, placebo-controlled Phase II study (Neuschwander-Tetri et al. 2015). One hundred forty-two and 141 patients were randomized to placebo or 25 mg of OCA, respectively, and treated for 72 weeks. The primary outcome of the study was improvement in liver histology defined as a decrease in NAS score by at least 2 points. A greater percentage of patients in the OCA arm compared to the control arm had improved NAS scores and histology scores regarding steatosis, hepatic inflammation, fibrosis, and hepatocyte ballooning. OCA reduced body weights and serum activities of ALT and GGT (Mudaliar et al. 2013). There was also a modest decrease in systolic blood pressure in OCA-treated patients. OCA increased serum activities of ALP and levels of LDL while decreasing levels of HDL. Contrary to the other Phase II study findings, fasting insulin and HOMA-IR were increased in OCA-treated patients. The most common side effect was pruritus (23.4% vs. 6.3% in placebo), which led to some patients receiving antipruritic mediation or temporary discontinuation of OCA.

Two Phase III trials are currently underway investigating the effects of OCA for the treatment of NASH. The REGENERATE trial is a multicentered, randomized, double-blinded, placebo-controlled trial that began in September 2015 and is currently recruiting patients. This study aims to follow 2,370 participants treated with either placebo, 10 mg of OCA, or 25 mg of OCA for 18 months. Participants will be non-cirrhotic NASH patients with fibrosis scores of 2 or 3. The primary endpoints under investigation are improvements in liver histology and progression to disease-

related events including common liver complications, HCC, liver transplantation, and death. As the FLINT and REGENERATE trials investigated and will investigate OCA in non-cirrhotic NASH, the REVERSE trial will study the effects of OCA in compensated cirrhotic NASH patients. This trial is a multicenter, randomized, double-blinded, placebo-controlled study that began in August 2017 and has a targeted estimated enrollment of 540 participants. Patients will be randomized to placebo, 10 mg of OCA, or 25 mg of OCA. The primary endpoint is the percentage of patients with histologic improvement of fibrosis by a score of 1 or more using the NASH Clinical Research Network scoring system. The expected completion dates of the REVERSE and REGENERATE trials are in 2020 and 2022, respectively. Several nonsteroidal FXR agonists have reached clinical trials. Compounds in this class are named using the drug suffix-*fexor* (i.e., tropifexor, nidufexor, turofexorate). The compound WAY-362450 described in the animal studies above was developed under the name FXR450 or turofexorate. A Phase I study using turofexorate was completed, but development was discontinued thereafter (Pharmaceuticals 2008). The compounds tropifexor (LJN452), nidufexor (LMB763), and EDP-305 have completed Phase I trials and are currently in Phase II trials (Novartis Pharmaceuticals 2018a, b, c; Enanta Pharmaceuticals 2018). A Phase II study was recently completed on GS-9674 (previously known as Px-104 and Px-102) and is currently under investigation in two additional Phase II studies (Gilead Sciences 2018a, b, c). GS-9674 is a close analog of GW4064 (Hambruch et al. 2012). See Table 1 for a summary of completed and ongoing trials with FXR agonists.

4.2 FGF19-Modified Protein

An analog of FGF19, NGM282, is currently in human clinical trials. A Phase I safety and tolerability study of NGM282 in adults has been completed as well as a 12-week-long Phase II safety, tolerability, and efficacy study in NASH patients (Harrison et al. 2018a; NGM Biopharmaceuticals 2013). Findings from the Phase II study mirrored results from preclinical animal studies. NGM282 decreased body weight and BMI. While no changes in hemoglobin A1c were observed, NGM282 reduced serum insulin levels and improved insulin sensitivity as evident by decreased HOMA-IR. NGM282 reduced absolute lipid content in the liver and reduced serum liver injury biomarkers ALT and AST. The levels of serum fibrosis biomarkers (pro-C3, PIIINP, and TIMP1) were reduced by NGM282. Fibrosis severity measured by multiparametric MRI was also decreased by NGM282. Histologic assessment of liver biopsies found that 84% of patients had improved NAS scores and 42% of patients had improved fibrosis stage. The primary difference of the findings from the Phase II study from preclinical animal studies pertains to serum lipid levels. NGM282 increased serum LDL levels in patients. However, concurrent treatment with a statin brought LDL levels back to baseline (Harrison et al. 2018b). Common adverse reactions were diarrhea (41% and 36%; 3 mg and 6 mg doses, respectively), abdominal pain (30% and 18%; 3 mg and 6 mg doses, respectively), and nausea (33% and 14%; 3 mg and 6 mg doses, respectively). Due to adverse

effects, 32% of patients treated with 6 mg of NGM282 had to interrupt or discontinue therapy (Harrison et al. 2018a).

As described previously, a FGFR1-βKL bi-specific activating antibody would be expected to have effects comparable to FGF21 and the extrahepatic effects of FGF19 mediated through FGFR1-βKL. NGM313 is an FGFR1-βKL bi-specific activating antibody currently in Phase I trials. NGM313 has already completed a Phase I trial in healthy adults and is now being studied in a Phase I trial in obese individuals (NGM Biopharmaceuticals 2017a, b).

5 Safety Concerns of FXR Agonist Therapy

5.1 Experiences with OCA

OCA is currently the only approved FXR agonist on the market and is approved for the treatment of PBC. In September 2017, just under a year and a half after its accelerated approval, an FDA safety communication was released regarding OCA (U.S. Food and Drug Administration 2017). This report described 11 cases of severe liver injury and 19 cases of death associated with OCA therapy. The communication described how these adverse outcomes appear to be due to excessive dosing, in particular frequency of dosing. In the OCA package insert, it is stated that in patients with moderate and severe liver injury, Child-Pugh classes B and C, the serum levels of OCA increase 4- and 17-fold, respectively (U.S. Food and Drug Administration 2017). Hence, dose adjustment is required for these patients; the medication is to be dosed weekly instead of daily (Ocaliva (obeticholic acid) 2018). In the 19 cases of death associated with OCA, 8 cases reported the cause of death. Of these eight cases, seven cases involved the daily dosing of OCA in patients with moderate and severe liver injury instead of the recommended weekly dosing. Of the 11 reports of severe liver injury induced by OCA, 6 were cases of patients with moderate or severe liver injury receiving daily dosing of OCA. The safety communication reminded healthcare providers to assess liver function in all patients before treating with OCA and to follow recommended dose adjustments. In February 2018, a follow-up safety communication was released stating that a black box warning was added to the OCA prescribing information (FDA 2018). This black box warning highlights the importance of screening liver function, properly selecting dose, and performing monitoring after initiation of therapy (Ocaliva (obeticholic acid) 2018). This communication urged prescribers to follow dosing on labeling, perform routine biochemical monitoring, recalculate Child-Pugh class, and adjust dosage accordingly when warranted (FDA 2018).

A second safety concern regarding OCA was identified during Phase II and Phase III NASH clinical trials (Mudaliar et al. 2013; Neuschwander-Tetri et al. 2015). In both trials, OCA treatment increased serum LDL levels while lowering HDL levels. As most NASH patients have underlying metabolic syndrome and higher rates of cardiovascular disease morbidity and mortality, these changes in serum lipid levels may lead to detrimental consequences. The ongoing Phase III REGENERATE trial

will study the effects of 18-month-long OCA therapy in a targeted 2,370 patients with NASH (Intercept Pharmaceuticals 2018b). OCA had beneficial effects on liver histology in NASH patients during the FLINT trial, and therefore the benefit of OCA may outweigh potential cardiovascular risks. It will be of interest to see how OCA effects the development of NASH and cardiovascular outcomes in the large REGENERATE trial and the risk-benefit of OCA treatment.

5.2 Carcinogenicity of FGF19 and Relevance of Animal Carcinogenicity Studies

In multiple mouse models, it has been demonstrated that activation of FGFR4/βKL by FGF19, but not FGF15 or NGM282, is carcinogenic (Zhou et al. 2014, 2017b; Wu et al. 2010; Luo et al. 2014). While FGF15 and FGF19 are orthologs, they only share 50% amino acid sequence homology (Nishimura et al. 1999; Xie et al. 1999). Additionally, FGF15 has an unpaired cysteine not present in the sequence of FGF19. It has been proposed that the unpaired cysteine in FGF15 forms an intermolecular disulfide bond leading to the formation of FGF15 homodimers. In nonreducing gels, it was shown that anti-FGF15 antibodies detect only FGF15 dimers, whereas in reducing gels anti-FGF15 antibodies detect only FGF15 monomers. In both nonreducing and reducing gels, FGF19 is detected as a monomer. This study proposed that FGF15 circulates as a homodimer and therefore may lead to different signaling outcomes than those induced by FGF19. The authors further speculated that the altered configuration of FGF15 is responsible for its lack of carcinogenicity (Zhou et al. 2017b). The stark differences in carcinogenicity of FGF15 and FGF19 raise the concern that there is a lack of animal model able to adequately assess the carcinogenicity of FXR agonists. If an FXR agonist is found to be non-carcinogenic in rodent models, one must consider if this is indeed due to lack of carcinogenic risk or due to the fact that rodents express FGF15 and not FGF19.

6 Summary

NASH is within the spectrum of NAFLD and is characterized by hepatic steatosis, hepatocyte ballooning, inflammation, and fibrosis and is associated with metabolic syndrome. Despite its high prevalence and severe health detriments, there is currently no approved therapy to treat NASH. Great effort has therefore been made to identify mechanisms underlying NASH pathogenesis and to develop efficacious therapies. One target identified to affect NASH development in animal models is the nuclear receptor FXR. Activation of FXR in multiple tissues and cell types attenuates the severity of the major characteristics of NASH. It is for this reason the development of FXR agonists has been an active area of research in the pharmaceutical industry and in academic research. There is currently one FXR agonist, OCA, already on the market for the treatment of PBC. OCA has completed two Phase II clinical trials for the treatment of NASH with two Phase III trials ongoing. There are

several other FXR agonists at various phases of clinical trials. As with most drug targets, FXR agonists have potential safety liabilities. In particular, safety concerns include pruritus, the worsened serum lipid profile in patients treated with OCA, the carcinogenic risk of FGF19, and the potential lack of relevant animal model for preclinical carcinogenicity studies. Despite these challenges, the development of FXR agonists provides hope for patients with NASH whose only treatment option currently is lifestyle modification and liver transplant. It will be of great interest in the upcoming years to see how FXR agonists perform in ongoing clinical trials and whether an FXR agonist becomes the first approved drug for the treatment of NASH.

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Targeting Bile Acid-Activated Receptors in Bariatric Surgery

Lili Ding, Zhipeng Fang, Yanjun Liu, Eryun Zhang, Tracy Huang, Li Yang, Zhengtao Wang, and Wendong Huang

Contents

Abstract

Bariatric surgical procedures, including Roux-en-Y gastric bypass and vertical sleeve gastrectomy, are currently the most effective clinical approaches to achieve a significant and sustainable weight loss. Bariatric surgery also concomitantly improves type 2 diabetes and other metabolic diseases such as nonalcoholic

L. Ding · E. Zhang
Department of Diabetes Complications and Metabolism, Diabetes & Metabolism Research Institute of City of Hope, Beckman Research Institute of City of Hope, Duarte, CA, USA

Shanghai Key Laboratory of Compound Chinese Medicines and The Ministry of Education (MOE) Key Laboratory of Standardization of Chinese Medicines, Institute of Chinese Materia Medica, Shanghai University of Traditional Chinese Medicine, Shanghai, China

Z. Fang · Y. Liu · W. Huang (✉)
Department of Diabetes Complications and Metabolism, Diabetes & Metabolism Research Institute of City of Hope, Beckman Research Institute of City of Hope, Duarte, CA, USA
e-mail: whuang@coh.org

T. Huang
Eugene and Roth Roberts Summer Student Academy, City of Hope, Duarte, CA, USA

L. Yang · Z. Wang
Shanghai Key Laboratory of Compound Chinese Medicines and The Ministry of Education (MOE) Key Laboratory of Standardization of Chinese Medicines, Institute of Chinese Materia Medica, Shanghai University of Traditional Chinese Medicine, Shanghai, China

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steatohepatitis, cardiovascular diseases, and hyperlipidemia. However, despite the recent exciting progress in the understanding how bariatric surgery works, the underlying molecular mechanisms of bariatric surgery remain largely unknown. Interestingly, bile acids are emerging as potential signaling molecules to mediate the beneficial effects of bariatric surgery. In this review, we summarize the recent findings on bile acids and their activated receptors in mediating the beneficial metabolic effects of bariatric surgery. We also discuss the potential to target bile acid-activated receptors in order to treat obesity and other metabolic diseases.

Keywords
Bariatric surgery · Bile acid · Diabetes · FXR · Obesity · TGR5

Abbreviations

BA	Bile acid
FXR	Farnesoid X receptor
NAFLD	Nonalcoholic fatty liver disease
NASH	Nonalcoholic steatohepatitis
RYGB	Roux-en-Y gastric bypass
T2D	Type 2 diabetes
TGR5	G protein-coupled bile acid receptor 1 (GPBAR-1, MBAR1, or TGR5)
VSG	Vertical sleeve gastrectomy

1 Bariatric Surgery: Introduction

Over the last few decades, the incidence of obesity has risen rapidly in both developed and developing countries, including the USA, where at least 33% of the population is classified as obese (Gregg and Shaw 2017; Higuchi and Kami 2017). A major complication of obesity is type 2 diabetes mellitus (T2D), a complex metabolic disease characterized by insulin resistance and the progressive failure of pancreatic β-cells (Abdelaal et al. 2017). To date, the effective treatments for obesity, T2D, and other related metabolic diseases are limited to a combination of diet, exercise, and medications. Unfortunately, patients who lose weight under these regimens frequently gain it back, and those treated with antidiabetic pharmacotherapies often experience unwanted side effects, including hypoglycemia, bone loss, and increased risk of certain cancers (Rubino et al. 2010; Puzziferri et al. 2014). Patients also often fail to maintain proper glycemic control and may experience life-threatening vascular complications (Gregg and Shaw 2017).

Due to a lack of successful pharmacotherapies, over 200,000 patients in the USA resort to bariatric surgeries each year to improve their obesity-related complications

and T2D (Schauer et al. 2017; Anker et al. 2016; Zaccardi et al. 2012). Several surgical procedures, involving various combinations of gastric resection and intestinal rearrangement, have been validated in rodents and humans: including adjustable gastric banding (AGB), ileal interposition (IT), duodenal-jejunal bypass (DJB), Roux-en-Y gastric bypass (RYGB), and vertical sleeve gastrectomy (VSG) (Liu et al. 2008; Stefater et al. 2012; Kohli et al. 2013a; Bayham et al. 2012; Lassailly et al. 2015). With the exception of AGB, in which a silicone band is used to control stomach volume, these surgeries involve the resection of the stomach and/or translocation of an intestinal segment. Until now, bariatric surgery has been the most effective treatment for sustainable weight loss, the remission or improvement of T2D, and nonalcoholic fatty liver disease (NAFLD) (Schauer et al. 2017; Anker et al. 2016; Zaccardi et al. 2012). Currently, RYGB and VSG have been the two most popular therapies for the treatment of obesity and the improvement of metabolic complications in clinic (Schauer et al. 2017; Arterburn and Courcoulas 2014). RYGB is a surgical procedure in which the stomach is divided into a small upper pouch and a much larger lower "remnant" pouch. Then, the small intestine is rearranged to connect to both. It has both restrictive and malabsorptive properties due to the combination of a small gastric pouch and total bypass of the duodenum and the proximal jejunum (Fig. 1a). VSG, in contrast, is a surgical procedure where approximately 80% of the stomach is removed along the greater curvature, which creates a gastric "sleeve" in continuity with the esophagus and duodenum (Fig. 1b). Among different bariatric surgeries, RYGB and VSG in particular have demonstrated striking

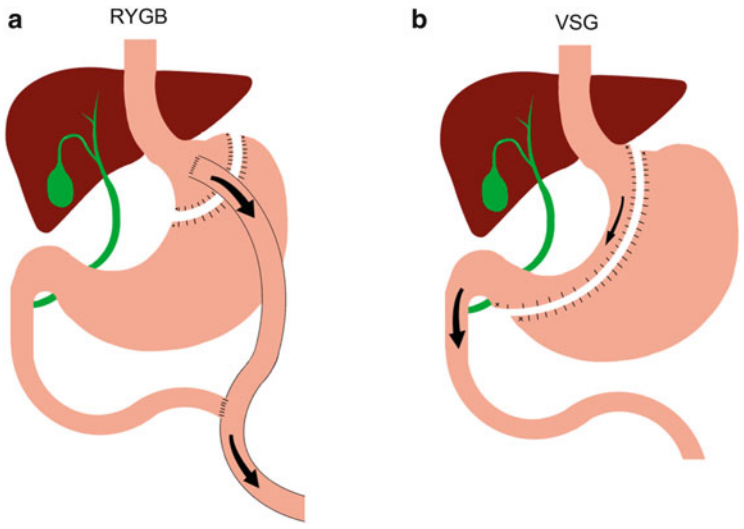

Fig. 1 RYGB and VSG: two typical types of bariatric surgery. (**a**) In RYGB, the stomach is divided into a small upper pouch and a much larger lower "remnant" pouch, and then the small intestine is rearranged to bypass the duodenum and the proximal jejunum. (**b**) In VSG, approximately 80% of the stomach is removed along the greater curvature. This creates a gastric "sleeve" in continuity with the esophagus and duodenum

results in terms of weight loss and glycemic control. In both procedures, a high percentage of obese T2D patients experience sustained weight loss and significant remission of T2D (Table 1). Interestingly, several studies have demonstrated early improvements in glucose tolerance prior to the loss of adipose tissue mass, suggesting that the anatomical changes associated with these bariatric surgeries may result in immediate systemic metabolic changes that can improve glucose homeostasis (Cummings et al. 2004; Kohli et al. 2015). In fact, bariatric surgery was recommended for the treatment of T2D in individuals with a body mass index (BMI) greater than 35 kg/m^2, according to the revised guidelines for the management of T2D from the 2nd Diabetes Surgery Summit in 2015 (Rubino et al. 2016).

Despite the overall success and improvement in bariatric surgeries, these clinical practices still remain invasive and risky. The bariatric surgery mortality rate has been reported to be between 0.3% and 2.0%, and there exists significant risk for serious, long-term side effects, including anastomotic leaks, severe infection, severe hypoglycemia, protein-calorie malnutrition, and deficiencies in iron and vitamins A, B_{12}, D, and E (Xanthakos 2009; Benotti et al. 2014). Moreover, the surgery is not routinely available to the low-income populations that are disproportionately affected by obesity and T2D. It is therefore imperative to better understand the molecular and physiological mechanisms by which bariatric surgery improves obesity and T2D. The astonishing effects of bariatric surgery have previously been attributed to the pure mechanical aspects of these procedures, such as restriction, malabsorption, and a reduction in food intake (Arble et al. 2015a). However, recent evidence from clinical and experimental animal models suggests that metabolic programming along the gastrointestinal tract and other organs is highly possible and essential. Among the many potential mediators, bile acids (BAs) and BA signaling seem to play an important role in modulating the beneficial effects of bariatric surgery (Ryan et al. 2014; Albaugh et al. 2017; Noel et al. 2016; McGavigan et al. 2017; Ding et al. 2016).

2 Bile Acids, Gut Microbiome, and Bariatric Surgery

Bile acids are the steroid acids transformed from cholesterol and are known for their important physiological functions required for the disposal of cholesterol and absorption of vitamins and fats (Monte et al. 2009). BAs are synthesized in the liver (Chiang 1998) and stored in the gallbladder. They are then secreted into the intestine when a meal is ingested, but 95% of BAs are reabsorbed and transported back to the liver through the portal vein, while about 5% of BAs are lost in feces. This process is known as enterohepatic circulation (Fan et al. 2015). Enterohepatic circulation maintains a constant pool of BAs that function as gastrointestinal (GI)-tract hormones to regulate glucose/lipid metabolism and other metabolic activities (Li et al. 2013; Wang et al. 2008; Fiorucci et al. 2009; Zhang et al. 2012; Keitel and Haussinger 2012; Schaap et al. 2014).

Table 1 Metabolic improvement after bariatric surgery

	RYGB	VSG	References
Metabolic benefits			
Body weight	↓	↓	Ding et al. (2016), Brolin et al. (1994)
Glucose homeostasis			
Fasting glucose	↓	↓	Roslin et al. (2012)
Insulin sensitivity	↑	↑	Chambers et al. (2011), Nosso et al. (2016)
Insulin secretion	↑	↑	Nosso et al. (2016), Douros et al. (2018), Arble et al. (2018), Salinari et al. (2013), Cummings et al. (2012)
Lipid homeostasis			
Plasma cholesterol	↓↓	↓	Stefater et al. (2011), Blanchard et al. (2018)
Plasma triglycerides	↓	↓	Stefater et al. (2011), Cazzo et al. (2014)
Plasma HDL	↑	↑	Cazzo et al. (2014), Davidson et al. (2017)
Plasma LDL	↓↓	↓ or ↔	Cazzo et al. (2014), Davidson et al. (2017), Benaiges et al. (2011, 2012), Hady et al. (2012)
Energy balance			
Calories intake	↓	↓	Arble et al. (2015b), le Roux et al. (2011)
Food aversions	Fat ↓	Fat ↓	le Roux et al. (2011), Chambers et al. (2012)
Food preference	Lower caloric	Lower caloric	le Roux et al. (2011), Wilson-Perez et al. (2013b)
Energy expenditure	↑↑	↑	Ding et al. (2016), Werling et al. (2015), Stylopoulos et al. (2009)
Bile acid			
Bile acid homeostasis			
Total bile acid pool	↑↑	↑↑	Myronovych et al. (2014b), Ahmad et al. (2013)
Primary bile acids	↑	↑	Tremaroli et al. (2015), Belgaumkar et al. (2016)
Secondary bile acids	↑↑	↑↑	Tremaroli et al. (2015), Albaugh et al. (2015)
Conjugated bile acids	↑ or ↔ or ↓	↑	Cummings et al. (2012), Myronovych et al. (2014b), Jahansouz et al. (2016), Simonen et al. (2012)
Taurine-conjugated bile acids	↓ or ↔	↑	Ding et al. (2016), Cummings et al. (2012), Myronovych et al. (2014b), Simonen et al. (2012), Spinelli et al. (2016)
Glycine-conjugated bile acids	↑	↔	Ding et al. (2016), Cummings et al. (2012), Simonen et al. (2012), Spinelli et al. (2016)

(continued)

Table 1 (continued)

	RYGB	VSG	References
Unconjugated bile acid	↑	↑	Spinelli et al. (2016), Bhutta et al. (2015)
12α-OH/non-12α-OH BA ratio	↓ or ↔	↓↓	McGavigan et al. (2017), Bhutta et al. (2015)
Fecal BA excretion	↑	↑	Ding et al. (2016), Bhutta et al. (2015)
Bile acid synthesis			
FXR	↓ or ↔	↑ or ↔	Ryan et al. (2014), Ding et al. (2016), Bhutta et al. (2015)
FGF19/FGF15	↑	↑	Ding et al. (2016), Sachdev et al. (2016)
SHP	↔	↓ or ↔	McGavigan et al. (2017), Perkins et al. (2014)
Cyp7a1	↓ or ↔	↓ or ↑ or ↔	Ding et al. (2016), Myronovych et al. (2014b), Simonen et al. (2012), Bhutta et al. (2015)
Cyp7b1	↑	↑	Ding et al. (2016), Flynn et al. (2015)
Cyp8b1	↓ or ↑ or ↔	↓↓	McGavigan et al. (2017), Ding et al. (2016), Myronovych et al. (2014b), Bhutta et al. (2015)
Cyp27a1	↓	↓ or ↔	Ding et al. (2016), Myronovych et al. (2014b), Bhutta et al. (2015)
Gastrointestinal hormones			
FGF19	↑↑	↑	Gerhard et al. (2013), Haluzikova et al. (2013), De Giorgi et al. (2015), Nemati et al. (2018)
GLP-1	↑↑	↑	Cummings et al. (2012), Hutch and Sandoval (2017), Dirksen et al. (2013), Nannipieri et al. (2013)
GLP-2	↑	↑	Cummings et al. (2012), Jorgensen et al. (2012), Romero et al. (2012)
GIP	↑ or ↔	↑	Nosso et al. (2016), Rhee et al. (2015)
Ghrelin	↑ or ↔	↓	Grayson et al. (2014), Santiago-Fernandez et al. (2017)
PYY	↑	↑	Cummings et al. (2012), Dirksen et al. (2013), Nannipieri et al. (2013)
CCK	↑	↑	De Giorgi et al. (2015), Rhee et al. (2015)
Oxyntomodulin	↑	N/A	Wu et al. (2013)
Leptin	↑	↑	Kalinowski et al. (2017), Stefater et al. (2010), Mokadem et al. (2015)
Change in gut microbiome	Yes	Yes	Flynn et al. (2015), Kaska et al. (2016), Arble et al. (2018), Anhe et al. (2017)

N/A indicates "not announced," ↓ indicates "mild decrease," ↓↓ indicates "major decrease," ↑ indicates "mild increase," ↑↑ indicates "major increase," ↔ indicates "no change"

Recent studies have demonstrated that both RYGB and VSG increase circulating BA concentrations (van Berge-Henegouwen and Hofmann 1983; Gerhard et al. 2013; Nakatani et al. 2009; Patti et al. 2009). These elevated BA levels could be sustained for many years post-surgery (Nakatani et al. 2009; Patti et al. 2009). To understand the roles of BAs in the post-surgical benefits of bariatric surgery, mouse models of bariatric surgery that recapitulate the human response are used widely, which permits the combination of genetic and pharmacological approaches to elucidate the mechanisms underlying the benefits of bariatric surgery. By taking advantage of those mouse models, BAs have been identified as potential mediators of the weight-independent effects of bariatric surgery, with respect to glucose homeostasis and insulin sensitivity. The mechanisms underneath these effects are currently of great interest.

Bile diversion, a newly developed bariatric surgical procedure in mice, is capable of elevating circulating bile acids through ligation of the common bile duct and anastomosis of the gallbladder to the ileum (GB-IL). It was enough to sustain the improvements in weight loss, glucose tolerance, and hepatic steatosis (Flynn et al. 2015; Kohli et al. 2013b), highlighting the significant contribution of BAs in mediating the effects of bariatric surgery. Apart from the alteration of total BA levels, the bariatric surgeries also change the composition of serum BAs (Liu et al. 2008; Stefater et al. 2012; Kohli et al. 2013a), which could be more important in obesity pathogenesis than the total BA levels. BA composition defines the hydrophobicity of BA pool, which affects the lipid absorption efficiency in small intestine. Haeusler et al. showed that the ratios of 12α-hydroxylated/non-12-α-hydroxylated BAs were associated with insulin resistance in humans (Haeusler et al. 2013). In another study involving human nonalcoholic steatohepatitis (NASH), the aggressive form of nonalcoholic fatty liver disease (NAFLD), BAs were found to be significantly increased in NASH patients than when they were in simple steatosis (Bechmann et al. 2013; Jiao et al. 2018). The ratio of CDCA was lower while DCA was significantly increased in NASH patients, while there was a comparable CA and UDCA ratio, suggesting that the elevated 12α-OH (CA+DCA)/12α-non-OH (CDCA +UDCA) ratio may highly correlate with the progress of NAFLD (Jiao et al. 2018). Surprisingly, the FGF19-FXR pathway was suppressed in NASH patients, indicating that the 12α-OH/12α-non-OH ratio of BAs, but not the BA pool size, was the major regulatory elements for this pathway (Jiao et al. 2018).

It has been well known that there is a dynamic interaction between BAs and gut microbiota (Fig. 2). Conjugated primary BAs can be deconjugated by microbiota in the intestine, which can be further de-hydroxylated at the 7α position by the bacteria to produce secondary BAs (Wahlstrom et al. 2017). On the other hand, BAs can also affect the growth of some specific microbiome such as *Clostridium difficile* in the intestine (Buffie et al. 2015). As expected, bariatric surgery such as RYGB dramatically alters the profiles of gut microbiome (Sweeney and Morton 2013). Bariatric surgery at least partially reverses obesity-associated microbial in obese individuals

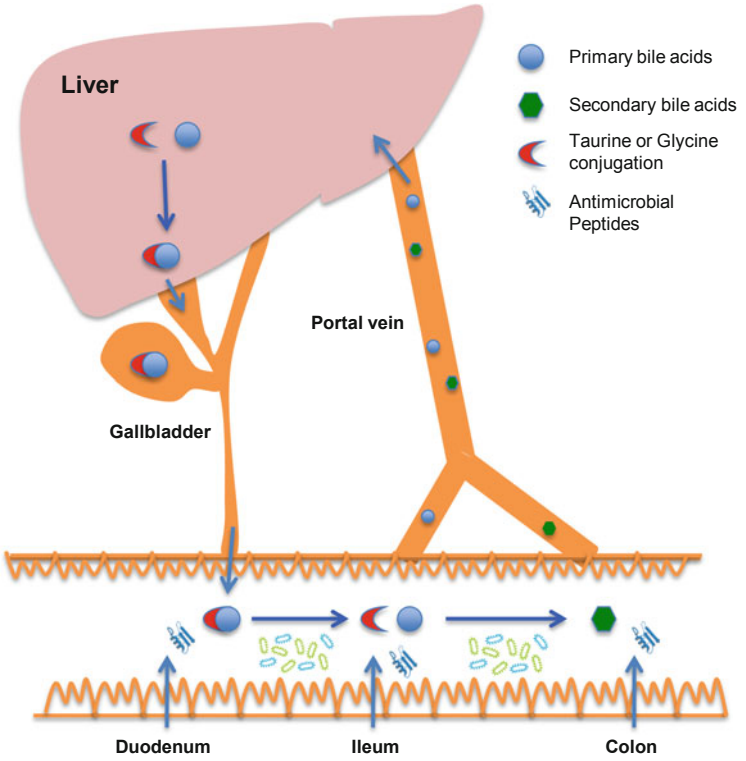

Fig. 2 Mutual interactions between bile acids and gut microbiota. The taurine- or glycine-conjugated primary bile acids enter the duodenum and are deconjugated by the gut microbiota. They can be further metabolized into secondary bile acids in the colon. Intestinal bile acids are reabsorbed and circulated back to the liver through the portal vein. On the other hand, bile acids may modulate antimicrobial peptides (AMPs) secretion from the intestine to alter the gut microbiota profile

(Liu et al. 2017). This is achieved by probably changing the gut microbiota of an obese individual to a profile that is commonly observed only in lean individual (Zhang et al. 2009; Li et al. 2011). Another report shows that VSG mice exposed to antibiotics, regardless of their specificity, have significantly increased subcutaneous adiposity and impaired glucose homeostasis without changes in food intake relative to control sham-operated mice, implicating that the intestinal microbiota is an important contributor to the metabolic improvement after surgery (Jahansouz et al. 2018). Together, the mutual interactions between BAs and gut microbiota may contribute significantly to the overall metabolic improvement, the detailed underlying mechanisms of which will be of great interest for future investigation.

3 BA Nuclear Receptor FXR and Bariatric Surgery

The hormonal roles of BAs in metabolic regulation make them attractive candidates as mediators for the beneficial effects of bariatric surgery. BAs act as signaling molecules to regulate lipid, glucose, and energy homeostasis by activating mainly the nuclear farnesoid X receptor (FXR or NR1H4) (Wang et al. 1999; Parks et al. 1999) and the cell membrane-associated G protein-coupled BA receptor 1 (TGR5, also known as GPBAR1 or MBAR1) (Staels and Fonseca 2009; Du et al. 2017; Akinrotimi et al. 2017). FXR is known as a primary bile acid nuclear receptor (Wang et al. 1999; Parks et al. 1999; Liu et al. 2016), which plays a central role in mediating the negative feedback regulation of BA synthesis (Li and Chiang 2015; Li et al. 2014) in both the liver and the small intestine.

Interestingly, FXR was recently identified to be a potential molecular target of VSG (Ryan et al. 2014). In the study conducted by Ryan et al., FXR was identified as a potential pathway altered by VSG in the distal small intestine. Mice with a systemic ablation of FXR gene were subjected to VSG along with wild-type controls. They found that, in contrast to the wild-type controls, the VSG-induced body weight loss, fat reduction, and improvement of glucose tolerance were almost completely absent in FXR knockout mice (Ryan et al. 2014). This is the first study to show that a single-gene knockout can abolish VSG effects in animals, highlighting the importance of BA signaling and a potential role of FXR in mediating the metabolic effects of bariatric surgery (Ryan et al. 2014). However, the interpretation of the results may be complicated due to the fact that the FXR knockout mice already have lower body weight and an enlarged BA pool size at the time of surgery, which may have prevented further weight reduction after surgery. Moreover, changes in plasma BAs and the expression of FXR after surgery were not shown in the study. Further studies will be required to better elucidate the exact roles FXR plays in metabolic improvement after bariatric surgery. Interestingly, there is also a correlation between BA-FXR signaling and the gut microbiota (Kaska et al. 2016). Normal mice transplanted with fecal microbiota transplants from RYGB-operated mice exhibit elevated BAs and a microbial population similar to "lean" mice (Liou et al. 2013). The relative abundance of certain bacteria, such as *Bacteroides* and *Roseburia*, is significantly altered in wild-type mice after VSG compared with the effects of sham surgery but remains in FXR knockout mice after surgery. Both FXR knockout and wild-type mice undergo a similar gut alteration after VSG, but only wild-type mice show a "lean" microbiota phenotype. This suggests that BA-FXR signaling may be a key to both changes in gut microbiota and associated weight loss and improvement in glucose metabolism (Ryan et al. 2014).

Although the results are interesting, there still remain some issues that need to be addressed. First, since FXR-deficient mice are both obesity resistant and insulin resistant, it is not an ideal model to examine the role of FXR in VSG-induced weight loss and glucose tolerance. Moreover, although bariatric surgery leads to an elevated level of BAs, how it affects FXR activation may be different between humans and rodents. In humans, the major primary BAs are CA and CDCA; however, in rodents,

most of CDCA is converted into MCA by the enzyme Cyp2c70 (Takahashi et al. 2016). CDCA is a potent FXR agonist while MCA is a potent antagonist of FXR. Therefore, the elevated MCA level after bariatric surgery would supposedly induce a much lower activation of the FXR-FGF15 (FGF19) pathway in rodents than in humans. Indeed, several studies in humans found that RYGB elevated the concentrations of BA and FGF19, meditating the improvement in glycemic control and diabetes remission (Gerhard et al. 2013; Sachdev et al. 2016; Murphy et al. 2018). There exists some debate though, as there is another study that suggests that the elevated FGF19 concentration is not responsible for the early improvement in glucose metabolism and insulin resistance after RYGB (Jorgensen et al. 2015). However, the expression levels of FGF15 (FGF19) in rodents are not reported in the above work (Ryan et al. 2014) or many other studies, leaving the debates unsolved. Meanwhile, the bile diversion surgery in obese mice is efficient for weight loss. The mice showed an elevated BA levels but reduced activity of the FXR-FGF15 signaling axis (Flynn et al. 2015). Therefore, the function of the FXR-FGF15 (Benotti et al. 2014) pathway or other FXR-related pathways in bariatric surgery needs to be further dissected; furthermore, other mouse models such as Cyp2c70-deficient mice should be utilized to mimic the BA composition in human.

So far, there is no clear evidence that shows whether bariatric surgery changes FXR activities, either through activation or suppression. Previous reports indicate that both FXR agonism and antagonism could have similar effects for improvement of hyperlipidemia (Ali et al. 2015; Lamers et al. 2014). Several FXR agonists such as obeticholic acid (OCA), EDP-505, and LMB-673 are currently under clinical trials to treat nonalcoholic steatohepatitis (NASH) (Friedman et al. 2018). Meanwhile, one of the FXR antagonists, guggulsterone, a natural product from the guggul tree, could promote the transformation from cholesterol to BAs, since it has been shown to decrease the hepatic and LDL cholesterol level in both humans and mice (Urizar et al. 2002). A recent study of the morbidly obese patients receiving another FXR antagonist, UDCA, demonstrated similar results as with guggulsterone (Mueller et al. 2015). However, they also found that neutral lipid accumulation in both liver and visceral white adipose tissue was induced with UDCA treatment. In addition, several other studies have identified that either glycine- or taurine-conjugated β-muricholic acids (MCA) are potent FXR antagonist (Sayin et al. 2013; Gonzalez et al. 2016; Jiang et al. 2015). Compared with tauro-β-MCA, which could be deconjugated by the bile salt hydrolase (BSH) in some gut microbiomes, Gly-β-MCA is resistant to BSH. It also functioned as a potent intestine-specific FXR antagonist to repress the ileum's ceramides production, thereby preventing/reducing obesity, IR, and hepatic steatosis in mice (Gonzalez et al. 2016; Jiang et al. 2015). It would be of great interest to validate whether Gly-β-MCA can work similarly in human patients in future studies. In a more recent work, the antihyperglycemic effect of metformin is also attributed to the increase of an intestinal FXR antagonist, glycoursodeoxycholic acid (GUDCA) (Sun et al. 2018).

Another baffling issue is in which tissue(s) FXR is required for surgical positive effects. FXR is expressed in several different organs, including the liver, small intestine, kidney, and adrenal glands, and may exert some tissue-specific functions in metabolic regulation. For example, activation of intestinal FXR was reported to control the transintestinal cholesterol excretion (TICE) in mice, in which cholesterol was transferred immediately from the blood into the intestinal lumen preceding the mice's defecation (de Boer et al. 2017). This pathway can contribute approximately 30% of the fecal sterol output in mice. On the other hand, activation of hepatic FXR by OCA increased the macrophage reverse cholesterol transport (RCT) and promoted fecal cholesterol excretion as well. Therefore, to further identify the detailed mechanisms of the FXR pathway during bariatric surgery, a tissue-specific FXR-deficient mouse model or a tissue-specific agonist such as intestinal agonist, fexaramine, must be taken into consideration. Bariatric surgeries such as RYGB make a significant anatomical rearrangement of the small intestine. Many recent studies indicate that the upper and lower small intestine may exert different functions in metabolic sensing and nutrient absorption (Bauer et al. 2018; Breen et al. 2013). Whether FXR has various functions in the duodenum, jejunum, and ileum is still unknown, which requires further researched.

4 BA Membrane Receptor TGR5 and Bariatric Surgery

In contrast to the nuclear receptor FXR, TGR5 is a plasma membrane-bound, G protein-coupled receptor for BAs. TGR5 is expressed in multiple tissues, including the liver, intestine, adipose tissue, and muscle and is also highly expressed in immune cells (Duboc et al. 2014). As signaling molecules, BAs regulate a variety of metabolic processes via TGR5 (Kuipers et al. 2014). TGR5 activation increases energy expenditure through modulating the activity of type 2 deiodinase (D2) and the subsequent activation of thyroid hormone in both brown adipose tissue (BAT) and muscle (Watanabe et al. 2006). TGR5 signaling also induces mitochondrial fission through the ERK/DRP1 pathway, further improving mitochondrial respiration (Velazquez-Villegas et al. 2018). Furthermore, the activation of TGR5 by its agonist INT-777 increases glucagon-like peptide-1 (GLP-1) secretion in enteroendocrine L cells of the intestinal epithelium (Thomas et al. 2009; Brighton et al. 2015), which can modulate insulin sensitivity and β-cell mass (Watanabe et al. 2006; Kuhre et al. 2016; Kumar et al. 2016), thereby improving glucose homeostasis (Thomas et al. 2009). It is also noted that TGR5 has both positive and negative effects in hepatic metabolism in the fasting and feeding response and hepatic steatosis. It has been demonstrated that $TGR5^{-/-}$ mice are protected against fasting-induced hepatic steatosis due to the increased GH-Stat5 signaling (Donepudi et al. 2017). However, on the other hand, postprandial activation of TGR5 by BAs is known to improve insulin secretion from β-cells via stimulating glucagon-like peptide-1 (GLP-1) secretion in enteroendocrine L cells (Katsuma et al. 2005). Besides FXR-FGF15/19, a direct effect of BAs via TGR5 also regulates gallbladder filling. A novel BA-TGR5-glucagon-like peptide-2 (GLP-2) axis provides a nutrient-mediated feedback from BA to regulate

meal-related gallbladder contraction (Yusta et al. 2017). Therefore, TGR5 provides a molecular link between bile acids, glucose homeostasis, and energy regulation.

Although a potential role of FXR in mediating VSG effects has been described, the detailed mechanisms still require further investigation (Ryan et al. 2014; Myronovych et al. 2014a); we and other laboratories have demonstrated that TGR5 is also required for the improved insulin sensitivity, anti-obesity, and antihyperglycemic effects of VSG in mice (McGavigan et al. 2017; Ding et al. 2016). Following VSG, concentrations of most unconjugated and taurine-conjugated BAs in serum were significantly increased and activated TGR5 signaling (Ding et al. 2016). Interestingly, the expression of TGR5 is also significantly increased after VSG. VSG also alters both BA levels and composition in mice, resulting in enhancement of TGR5 signaling in the ileum and brown adipose tissues. These increases subsequently stimulated intestinal GLP-1 release and induced TGR5-D2-UCP1 signaling which may contribute to an increase of energy expenditure and physical activity, concomitant with sustained weight loss, as well as the improvement of fatty liver, glucose control, and insulin resistance in obese mice post-VSG (Ding et al. 2016). Similarly, McGavigan AK et al. showed that VSG-mediated fasting glycemia improvement, glucose tolerance, and glucose-stimulated insulin secretion were abolished in TGR5-KO mice (McGavigan et al. 2017). They further demonstrated that TGR5-dependent regulation of hepatic Cyp8b1 expression might contribute to TGR5-mediated shifts in the circulating BA pool after VSG (McGavigan et al. 2017). These two studies suggest that TGR5 may be a molecular target of bariatric surgery (McGavigan et al. 2017; Ding et al. 2016). However, future questions remain as to how VSG activates TGR5 and its major downstream effectors. Moreover, bile acid signaling through TGR5 is not required for the beneficial effects of RYGB in the mouse, suggesting RYGB and VSG may achieve their similar beneficial effects through different mechanisms (Hao et al. 2018). GLP-1 is a powerful hormone whose ability to stimulate insulin secretion and glucose clearance BAs may activate ileal TGR5-mTORC1 signaling to increase GLP-1 production after RYGB (Ding et al. 2016; Zhai et al. 2018). However, it has been reported that both GLP-1 receptor (GLP-1R) and GLP-2 receptor (GLP-2R) are not required for the beneficial effects of bariatric surgeries (Wilson-Perez et al. 2013a; Patel et al. 2018). It's also possible that GLP-1/2 contribute to the bariatric surgery effects by the independent ways of GLP-1/2 R (Tomas and Habener 2010; Nuche-Berenguer et al. 2010).

Whereas the multiple metabolic functions of TGR5 agonist were identified, the application for drug development has been hindered by side effects including inhibition of gallbladder emptying and other syndrome. Thus, the tissue-specific agonism of TGR5 attracts much attention; it has been reported that intestinal TGR5 agonism improves hepatic steatosis and insulin sensitivity in Western diet-fed mice (Finn et al. 2019). Currently, most of the mechanistic studies provide evidence that BAs and their receptors regulate various metabolic processes in bariatric surgery (Ryan et al. 2014; McGavigan et al. 2017; Ding et al. 2016). Essentially, BAs can either directly or indirectly affect food intake, energy expenditure, and glycemic control through acting on TGR5, which can then exert their actions on a wide range of tissues including the hypothalamus (Li and Chiang 2014) (Fig. 3).

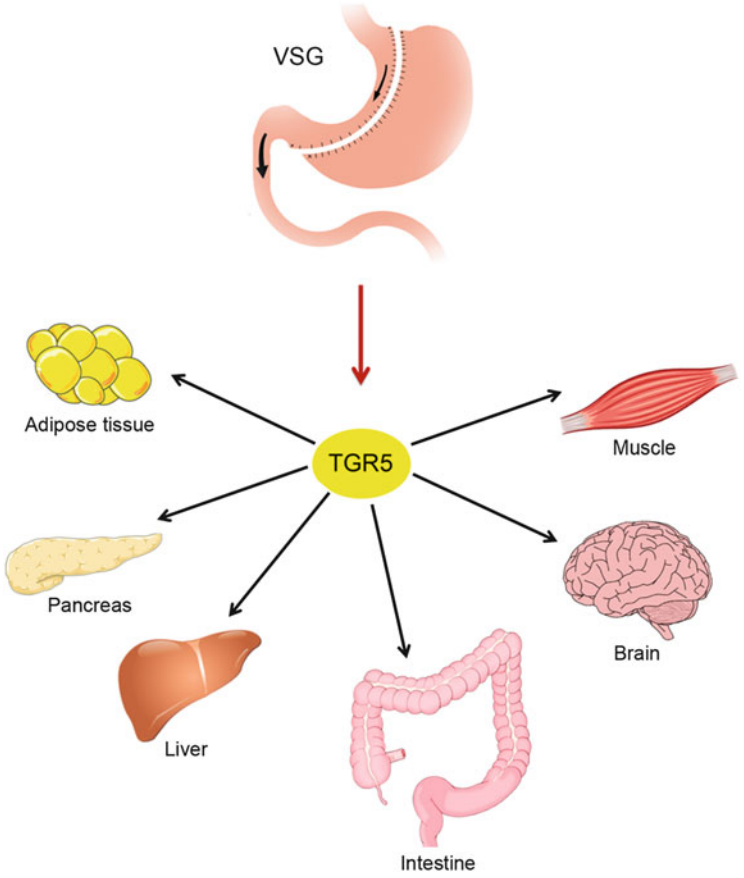

Fig. 3 TGR5 as a potential molecular target of VSG. VSG alters BA levels and composition as well as the gut microbiota. The activation of TGR5 after VSG may lead to metabolic changes in multiple tissues, including the intestinal GLP-1 secretion, inhibition of appetite, stimulation of the insulin secretion, and increase of energy expenditure

5 Perspectives

There have been significant advances in the last decade to better understand the potential mechanisms of bariatric surgery in treating obesity, as well as the obesity-related comorbid conditions. The surprising success of surgery in manipulating the GI tract to effectively alleviate obesity and T2D prompts us to reconsider the previously neglected roles of the gut in nutritional sensing and metabolic regulation. Furthermore, bariatric surgery provides an ideal model to seek novel insights into the

etiology and explore the fundamental mechanisms of obesity, diabetes, and other related metabolic diseases, which will eventually aid in developing new therapeutic strategies.

Although BAs are best known for their important lipid-solubilizing role in the assimilation and absorption of fat and other nutrients in the intestine, they now appear as key signaling molecules in modulating the effects of lipid metabolism, glucose metabolism, and gut microbiota regulation after bariatric surgery. Given their robust downstream effects in different metabolic tissues, both FXR and TGR5 may mediate some of the positive metabolic effects of bariatric surgery. Therefore, further understanding the roles of FXR and TGR5 in regulating metabolism will provide novel insights into the molecular mechanisms underlying the metabolic effects of bariatric surgery. At the same time, small molecules targeting BA receptors, as well as BA analogs and BA sequestrants, may provide new drug candidates in fighting against obesity and its related metabolic diseases.

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