

## Review Article

# On the Pharmacology of Farnesoid X Receptor Agonists: Give me an “A”, Like in “Acid”

**Eva Hambruch, Olaf Kinzel, and Claus Kremoser**

*Phenex Pharmaceuticals AG, Waldhofer Str. 104, 69123 Heidelberg, Germany*

Corresponding Author: Claus Kremoser; email: [claus.kremoser@phenex-pharma.com](mailto:claus.kremoser@phenex-pharma.com)

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**Abstract.** The Farnesoid X Receptor (FXR) has recently moved into the spotlight through the release of clinical data using Obeticholic Acid, an FXR agonist, that demonstrated effectiveness of this bile acid-like drug in patients with Primary Biliary Cirrhosis and Non-alcoholic Steatohepatitis (NASH). FXR holds the promise to become an attractive drug target for various conditions, from Non-alcoholic Fatty Liver Disease (NAFLD), NASH, liver cirrhosis, portal hypertension and a variety of cholestatic disorders to intestinal diseases including inflammatory bowel disease and bile acid diarrhea. Despite the wide therapeutic potential, surprisingly little is known about the pharmacology, pharmacokinetics and tissue distribution properties of drugs targeting FXR. Are tissue specific FXR agonists preferable for different indications, or might one type of ligand fit all purposes? This review aims to summarize the sparse data which are available on this clinically and pharmacologically relevant topic and provides a mechanistic model for understanding tissue-specific effects *in vivo*.

**Keywords:** Farnesoid X Receptor; pharmacology; liver transport of bile acids; acidic drug; Obeticholic Acid

## 1. Introduction

The Farnesoid X Receptor (FXR, NR1H4) was characterized as a member of the nuclear hormone receptor superfamily in 19 [1]. The original finding that FXR binds farnesol and other isoprenoid-type lipophilic ligands was superseded by the discovery that bile acids (BAs), most potently chenodeoxycholic acid and its conjugates, are the natural activators of this receptor [2–4]. FXR is a type II nuclear receptor which binds its cognate response elements (preferably an IR1 motif) as an obligate heterodimer complex with the Retinoid X Receptor (RXR) [1, 5]. This heterodimer complex is permissive for both partners: transcription of FXR/RXR target genes can be

initiated by 9-*cis*-retinoic acid or similar rexinoids as well as by selective FXR agonists.

FXR is expressed throughout the entire gastrointestinal tract from the esophagus to the rectum, and in the liver, the gallbladder, the kidneys and the adrenal glands [6, 7]. FXR expression has also been reported in the vascular endothelium, in the mammary gland, in macrophages and in adipose tissue, although these findings have not been widely confirmed [8–12]. The relevance of BAs as the natural ligands for FXR is corroborated by the observation that intestinal FXR activation with attendant FGF15/19 induction, together with hepatic FXR activation, synergistically controls the conversion of cholesterol into

BAs in the liver and their export into the bile [13, 14]. Beyond regulating bile acid metabolism in a feedback inhibition loop, FXR is involved in the homeostasis of several other metabolic parameters and pathways including liver and blood triglycerides, cholesterol and lipoprotein metabolism, glycogen synthesis and gluconeogenesis [15–17]. FXR activation or inhibition has an impact on the intestinal microbiome which in turn changes BA patterns and degree of conjugation. In connection with its direct metabolic effects and its indirect effects via modulating the intestinal microbiome, FXR might also be an attractive target for the treatment of obesity [18, 19]. FXR is also directly involved in the control of local vasotone via modulators such as hydrogen sulfide, asymmetric dimethylarginine (ADMA, an inhibitor of endothelial NO synthase) and endothelin-1 [20–22].

Beyond its fundamental role in metabolism, FXR impacts various genes involved in cell proliferation, with a physiological function in liver regeneration [23, 24]. Clinical expectations for FXR as a beneficial drug target are also based on findings that FXR upregulates genes involved in hepatoprotection (e.g., Glutathione Peroxidases, and Glutathione S-Transferases). FXR was also found to control xenobiotic drug metabolism and export (certain UDP-Glucuronyl-Transferases and canalicular bile acid and drug transporters) [25–28].

An as yet underexplored area is the involvement of FXR in the control of the immune system and of the intestinal microbiome and barrier function. It is clear that FXR influences the expression of immunomodulatory genes, but the outcome cannot be simply described as pro- or anti-inflammatory. Whereas some papers highlight the anti-inflammatory action of FXR on dampening NF-kappaB dependent transcription of proinflammatory cytokines in hepatocytes [29, 30], others have shown that FXR upregulates inducible nitric oxide synthase (iNOS), IL-18 and related factors that typically enhance the immune defense in the intestine [31, 32]. FXR is further involved in complex interactions with the gut microbiome, fostering production of endogenous antimicrobial peptides such as defensins and cathelicidins on one hand but also being antagonized in the intestinal epithelium by the hydrophilic bile acid Tauro-beta Muricholic Acid (T-b-MCA) [33, 34]. Intestinal FXR activation also prevents translocation of bacteria from the intestinal lumen into the peritoneum by upregulating tight junctions in the intestinal epithelium and likely also by changing the composition of the gut microbiome [31].

*1.1. FXR ligands: Bile acids, bile acid derivatives and synthetic FXR agonists.* Since the original papers in 1999, there have been more than 1300 publications on FXR, including dozens that describe different types of FXR modulating ligands – both natural (endogenous or external) and synthetic [reviewed in [35, 36]]. The prototypic FXR

agonist, based on the bile acid scaffold of the most potent natural activator CDCA, is 6-Ethyl-CDCA, also termed INT-747 or obeticholic acid (OCA) [[37], see Figure 1]. This compound was discovered by scientists at the University of Perugia and GlaxoSmithKline as early as 2002. GSK discontinued the program and OCA was licensed by Intercept Pharmaceuticals. The New York-based company developed this semi-synthetic bile acid clinically and conducted pivotal and successful phase II and III studies in patients with Primary Biliary Cirrhosis (PBC). PBC is a severe cholestatic liver disorder characterized by pruritus and progressive loss of liver function which can ultimately result in a need for liver transplantation. OCA decreased liver enzymes such as AST, ALT, GGT and alkaline phosphatase, in particular, as pivotal biomarkers of PBC progression in short (up to 12 weeks) or long (up to 72 weeks and longer, with follow up still ongoing) term clinical trials [38].

Interest in OCA was further reinforced by a phase IIb study in patients with NASH. The study was prematurely terminated upon interim analysis of approximately half of the 280 patients that were originally envisaged, in which OCA demonstrated convincing effectiveness, given at 25 mg, once a day for 72 weeks. OCA decreased the NAFLD activity score (NAS) in all three dimensions (steatosis, hepatocyte ballooning and inflammation) independently and in composite, and further showed a significant reduction in liver fibrosis based on histopathological analysis [39]. More recent data from a Japanese phase II trial testing OCA in NASH patients from Intercept's collaboration partner Dainippon Sumitomo failed to reach the endpoint of lowering the NAS by  $\geq 2$  units, but confirmed a trend in beneficial effects.

No other FXR agonist has reached the clinical maturity of OCA. As early as 1999, scientists from GlaxoSmithKline filed a patent application for the potent and selective FXR agonist GW4064, described as a “chemical tool” in a subsequent publication [40]. GW4064 was widely used to test for pharmacological effects of FXR agonists in various cell culture and animal models of liver and gastrointestinal diseases, but it never entered the clinic. WAY-362450 is a fully synthetic and potent FXR agonist, structurally completely independent from GW4064 (see Figure 1, [42]). This compound, abbreviated as FXR-450, was in-licensed by Wyeth from Exelixis, who in turn had obtained it through acquisition of X-ceptor Pharmaceuticals, a spinout from Ligand Pharmaceuticals and the Salk Institute, both located in San Diego, U.S.A. FXR-450 was taken into phase I studies but further development was abandoned for unknown reasons. Challenges in formulation were mentioned in a subsequent publication, which also identified derivatives with improved pharmacokinetic properties [43]. FXR-450 has also demonstrated effectiveness in animal models of dyslipidemia and NASH [44, 45].

PX20606 (Px-102) is a close derivative of GW4064 and was synthesized in our labs at Phenex Pharmaceuticals [see

Figure 1 and [41]). The compound is an analogue of GW4064 with a cyclopropyl ring replacing the stilbene double bond. Although this did not result in an increase in aqueous solubility, PX20606, its predecessor PX20350 and related structures showed much higher liver exposures and enhanced FXR liver transcriptional responses compared to OCA and GW4064 upon oral dosing in rodents [41]. The massively increased liver transcriptional response of PX20606 and PX20350 was not a function of better plasma bioavailability of these compounds, but of improved target organ (e.g., liver) exposure and residence time (see [46] for relevance of drug residence time).

Beyond OCA, different groups from the University of Perugia have synthesized further bile acid derivatives, mostly of CDCA, with differing degrees of potency and selectivity against the two main bile acid receptors FXR and TGR5 (or GPBAR1) - the latter representing a member of the family of membrane-bound G-protein coupled receptors (GPCRs) [47, 48].

Fexaramine was discovered by the groups of K. C. Nicolaou and R. Evans in 2003 [49, 50]. Like GW4064, it derives from a screening effort with further optimization for FXR binding and transactivation potency. Recent oral studies in rodents revealed the FXR agonism of fexaramine was limited to the intestine, but that beneficial metabolic pharmacodynamic effects resulted [51]. This raises the question of whether intestinally limited FXR agonism might be sufficient to generate the majority of the beneficial clinical effects attributed to FXR agonists. However, it should be noted that there are publications that claim to achieve beneficial effects with just the opposite, Glycine-beta-muricholic acid, acting as an intestine-selective FXR antagonist [104]. The final word in this debate about what intestinal FXR contributes to the overall metabolic effects is not spoken.

*1.2. Pharmacokinetic properties of FXR agonists and bile acid transport.* In contemporary drug discovery it is axiomatic that structural and physicochemical properties should be addressed so as to ensure adequate oral absorption, as assessed by plasma exposure. C. Lipinski formulated his famous “rule of five”, based upon an empiric analysis of successful oral drugs, in order to guide towards an optimal balance of aqueous solubility and membrane permeability [52]. In this context it is further assumed that passive flux through cell membranes ensures tissue penetration. Pharmacologists then estimate receptor occupancy (and hence functional activation/inhibition) through combining *in vitro* potency data with *in vivo* plasma or target tissue concentrations, correcting for the degree of protein (or tissue) binding. A simple formula for this approximation of a drug’s activity is therefore:

$$[C]_{\text{eff}} = [C]_{\text{det}} \times F \quad \text{with} \quad F = [C]_{\text{free}} / [C]_{\text{bound}}$$

with

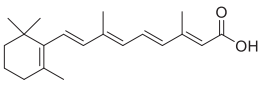
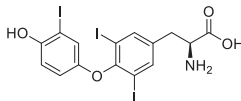
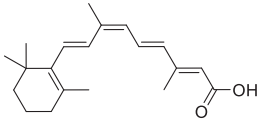
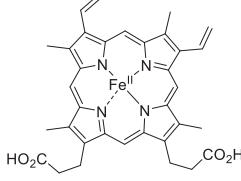
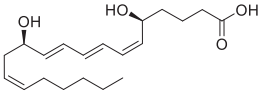
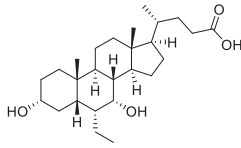
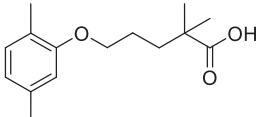
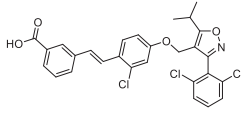
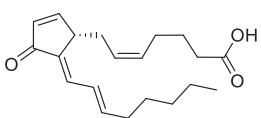
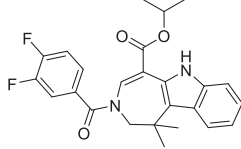
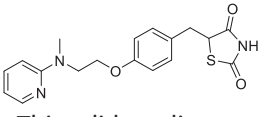
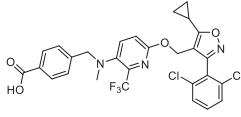
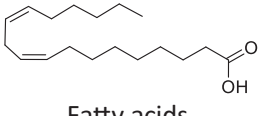
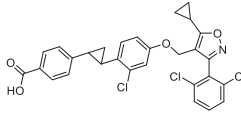
$[C]_{\text{eff}}$  = effective drug concentration in the observed compartment.

$[C]_{\text{det}}$  = drug concentration determined from bioanalytics.

$[C]_{\text{free}}$ ,  $[C]_{\text{bound}}$  = concentration of free and protein bound drug in a binding assay that is representative of the respective compartment. However, serum or plasma binding is widely used as a general surrogate for this.

Nuclear receptors, in general, have a tendency to bind small lipophilic ligands, and lipophilic ligands have an intrinsically high affinity for serum proteins [53]. Serum albumin typically also exhibits high affinity for acidic compounds, and BAs (as natural FXR ligands) are acidic either in their free form or as glycine or taurine conjugates. The terminal acidic moiety plus the presence of one to three hydroxy groups account for the amphiphilicity of BAs, which is the basis of their biochemical function as nutrient detergents [54]. The type II nuclear receptors heterodimerize with RXR, and many members of this subclass are known to sense fatty acids and derivatives or other lipophilic ligands, which are typically present in micro- to millimolar cellular concentrations (as opposed to steroids, which typically activate their cognate receptors at nanomolar concentrations) [53]. Many pharmacodynamically potent and clinically effective synthetic ligands for such Type II nuclear receptors, such as retinoids (RAR ligands), fibrates (PPARalpha ligands) or thiazolidinediones (PPARgamma ligands), are similarly lipophilic and acidic in nature (see Figure 1 for a list of acidic nuclear receptor ligands). Consistent with this general principle, the FXR ligands that have reached the clinic or have demonstrated activity in animal models typically bear an acidic moiety (OCA, GW4064, PX20350, PX20606) or at least contain a “buried” acid function in the form of an ester (FXR-450). With AGN34 there is even an FXR antagonist that contains a carboxylic acid function [103]. Carboxylic acids are not necessarily desired by medicinal chemists and other drug discovery scientists since they are prone for active membrane transport and can be metabolized to acyl glucuronides, potentially dangerous reactive metabolite species [55]. But they seem to have an impact on the *in vivo* efficacy of a FXR-targeted drug.

For FXR agonists that are natural BAs, BA derivatives (OCA) or synthetic ligands modeled after BAs (GW-4064, PX20606) there is another important aspect to consider in the context of target tissue activity in the liver. It is well established that conjugated BAs (which constitute 50–70% of the total pool in humans) are dependent on active transport mechanisms for membrane permeability. Several bile acid transmembrane proteins have been identified, cloned and sequenced over the last three decades [56, 57]. They are either Solute Carrier proteins (SLCs, [58]), a large and widespread family of transporters of various endogenous and xenobiotic small molecules, or ABC (=ATP-binding cassette)-transporters. ABC transporters, as the name implies, are unidirectional and energetically driven by ATP hydrolysis, whereas SLCs facilitate transport along an electrochemical

Nuclear Receptor	Ligand	[N/S]* Referen-ces	Nuclear Receptor	Ligand	[N/S]* Referen-ces
RAR	 all-trans-retinoic acid	N [86]	TR $\alpha$ / $\beta$	 Triiodothyronine (thyroid hormone)	N [94, 95]
RXR	 9-cis-retinoic acid	N [87]	Rev-erba/ $\beta$	 Heme	N [96]
PPAR $\alpha$	 Leukotriene B4, Fatty acids	N [88]	FXR	 [6-Ethyl]- Chenodeoxycholic Acid	N/S [97]
PPAR $\alpha$	 Fibrates, e.g. Gemfibrozil	S [89]	FXR	 GW4064	S [98]
PPAR $\gamma$	 15-deoxy- $\delta$ -12,14-PGJ <sub>2</sub>	N [90, 91]	FXR	 WAY-450	S [99]
PPAR $\gamma$	 Thiazolidenediones, e.g. Rosiglitazone	S [92]	FXR	 PX20350	S [100]
HNF4 $\alpha$	 Fatty acids, e.g. Linoleic acid,	N [93]	FXR	 PX20606	S [39]

[N/S]\* = natural (N) or synthetic (S) ligand

Figure 1: Acidic Nuclear Receptor Ligands.



gradient, or through symport or antiport of another solute (often  $\text{Na}^+$  or  $\text{H}^+$ ).

Active transport provides the basis for the enterohepatic cycling of BAs - i.e. very efficient (95–99%) intestinal reuptake (mostly in the terminal ileum), import into the liver, and active excretion from the hepatocyte into the lumen of the bile. During the latter step, BAs are sequestered up to 1000-fold. This concentration gradient is maintained by the high degree of conjugation in most species. As sulfonic acids, the taurine conjugates bear a permanent charge that prevents transmembrane diffusion, and although the glycine conjugates can potentially be protonated under physiological conditions this is specifically minimized in the bile by active bicarbonate secretion from cholangiocytes leading to a slightly alkaline milieu. This “bicarbonate umbrella” consequently protects the cholangiocyte epithelial lining from passive diffusion of BAs and membrane solubilization by these potent natural detergents [59]. However, this protective mechanism through bicarbonate alkalization of the bile only applies to humans since other species largely have only taurine amide conjugates which are permanently negatively charged anyway.

Active transport and containment mechanisms therefore serve to limit the exposure of BAs to the cytoplasmic or membranous compartments of cells. For enterocytes, it is well established that upon uptake from the intestinal lumen via the ileal bile acid transporter (IBAT, also called apical sodium-dependent bile acid transporter, ASBT), BAs are bound to proteins such as the ileal bile acid binding protein (IBABP). IBABP is a transcriptional target gene of FXR, such that agonism generates higher levels of the binding protein and lessens free bile acid concentrations inside the cell [60, 61]. IBABP escorts the passage of BAs from the luminal side until they are effluxed out of the enterocyte by the basolateral specific solute carriers  $\text{OST}\alpha$  and  $\beta$  following their concentration gradients [62].

What are the corresponding phenomena associated with transhepatic flux of BAs, from the blood-purged sinusoids towards the bile canaliculi? Most recent reviews implicitly assume a kind of “swimming pool” model for transhepatic bile acid flux: Basolateral (i.e. sinusoidal) bile acid uptake SLC transporters (OATPs for unconjugated BAs, NTCP for conjugated ones) pump BAs from the space of Disse into the hepatocyte, and on the canalicular side the ABC-type transporter BSEP translocates the majority of BAs into the bile against a steep concentration gradient. As for enterocytes, intracellular (putatively cytosolic) bile acid binding proteins such as liver-fatty acid binding protein (L-FABP) [63], certain glutathione-S-transferases (GSTs, [64]) and 3- $\alpha$ -hydroxysteroid dehydrogenase (3- $\alpha$ -HSD, [65]) are believed to bind the amphiphilic BAs and prevent them from causing damage through their potent detergent properties.

However, this simple picture of active bile acid transmembrane uptake, passive diffusion through the hepatocyte by means of BA-binding proteins, and active transmembrane

BA excretion neglects some important findings implicating more complex transport mechanisms. These findings are as follows:

1.2.1. BAs travel through organelles in a controlled manner. Unconjugated BAs are conjugated by enzymes that reside within peroxisomes. Rembacz et al. showed that cholic acid shuttles through peroxisomes to be conjugated by the residing enzymes Bile Acyl:CoA Synthetase (BACS) and Bile Acyl CoA:Amino acid N-acyltransferase (BAAT). While the import into peroxisomes might occur by passive transmembrane diffusion or a flip flop mechanism, the question of how the resultant tauro conjugate is transferred from the peroxisome and ultimately into the bile is still unanswered [66].

1.2.2. BAs use different paths across the hepatocyte depending on their hydrophobicity. Several papers published from the mid-1980s to the late 1990s provide evidence that for BAs, depending on their degree of hydroxylation (i.e., their hydrophilicity/hydrophobicity), different transcellular routes through the hepatocyte must exist [reviewed in [67, 68]]. Taurocholic acid appears to access a kind of “fast transit” route that involves certain tubulovesicular structures that can be labelled by horse radish peroxidase and fluorescent conjugated BAs [69]. Some authors claim the involvement of microtubule-dependent vesicular transport in the transcellular movement of cholic acid and derivatives [70, 71]. Other publications suggest that the more hydrophobic a bile acid is, the less likely it is that it is subject to these transcellular shunting routes – instead being subject to capture by intracellular binding proteins, or to association with other membranous structures [[72], reviewed in [68]]. Hydrophobic BAs such as lithocholic acid are believed to be transported into the perinuclear smooth endoplasmic reticulum, and are further transported to the canalicular membrane at a much slower rate than their more polar counterparts. For the purposes of this article, we refer to this as the “nuclear” route. Notably, it should be remembered that the smooth ER is a protrusion of the nuclear envelope membrane. There are initial reports that nuclear receptors might use the nuclear envelope membrane as a “resting place”, and that initial encounter with activating ligands might occur here [reviewed in [74]].

Beyond just binding BAs, the aforementioned L-FABP might have a role in delivering BAs to the “nuclear” route. L-FABP was found to bind several anionic lipophilic molecules, including BAs (with a preference for hydrophobic ones), fatty acids and cholesterol sulphate, but not cholesterol itself [75]. A naturally occurring amino acid variant (T94A) changes binding affinities for amphiphilic ligands and has even an impact on the pharmacological properties of fibrates [76]. The L-FABP knockout mouse shows significant alterations in cholesterol and bile acid metabolism as well as changes in the expression of nuclear receptors including LXRA and FXR [77]. L-FABP interacts directly with PPAR $\alpha$  and

HNF4 $\alpha$ , both nuclear receptors which are known to bind fatty acids [78, 79]. Addition of L-FABP to cultured cells expressing HNF4 $\alpha$  potentiates its transcriptional activity. Together with the findings that nuclear receptors might be tethered to the inner nuclear envelope and “wait” there for incoming amphiphilic ligands, this is suggestive of an explanation for the nuclear delivery of amphiphilic ligands that modulate nuclear receptors.

Figure 2 shows a working hypothesis of the putative intracellular routes that different types of BAs might use to cross the hepatocyte and how these routes might impact the interaction with FXR.

This difference in trafficking is understandable physiologically, for hydrophobic and unconjugated BAs are more cytotoxic than their conjugated or hydrophilic counterparts and according to our model would be subject to the “nuclear” route, leading to transcriptional activation of FXR. The hepatic FXR transcriptional repertoire provides for hydroxylation, conjugation and export, thereby providing a hepatoprotective effect.

*1.3. Why is the bile acid intracellular transport so relevant for FXR agonist pharmacology?* OCA, the first late stage clinical FXR agonist, is a bile acid - just not a natural one. Like its natural counterparts it is subject to bile acid transport and conjugation, and this has substantial impact on its pharmacokinetics and -dynamics. In mice and rats, OCA is conjugated in the liver and then undergoes efficient enterohepatic circulation, and there is a sharp asymmetry between serum and liver concentrations of OCA and its main rodent metabolite, Tauro-OCA (T-OCA). Roda et al. reported a 4500 times higher concentration of total OCA in the liver (610  $\mu\text{mol/g}$  liver) compared to plasma (0,135  $\mu\text{M}$ ) after intraduodenal infusion of OCA at 25mg/kg, with 25% of the total as T-OCA in plasma, and 90% of the total as T-OCA in the liver [80]. We found that after repeated dosing in mice at doses of 10 mg/kg, total OCA reached 1,4  $\mu\text{M}$  in the liver, with T-OCA constituting approximately 98% of this pool [41].

For OCA's natural prototype, CDCA, Parks et al. demonstrated that the tauro conjugate had similar if not superior FXR activity and was dependent on the expression of bile acid transporters (e.g., NTCP or ASBT) for cellular uptake *in vitro* [2]. The same is true for OCA and T-OCA (unpublished results), and so the finding that the massive hepatic accumulation of OCA and especially T-OCA *in vivo* does not result in major FXR-dependent transcriptional changes in mouse liver is puzzling [41], especially as at these therapeutic doses FGF15 or IBABP induction by OCA can still be observed in the intestine. The rapid extraction of OCA from the blood into the liver and the massive liver vs. blood accumulation of T-OCA demonstrates that active transport and sequestration mechanisms are at work that are not fully elucidated.

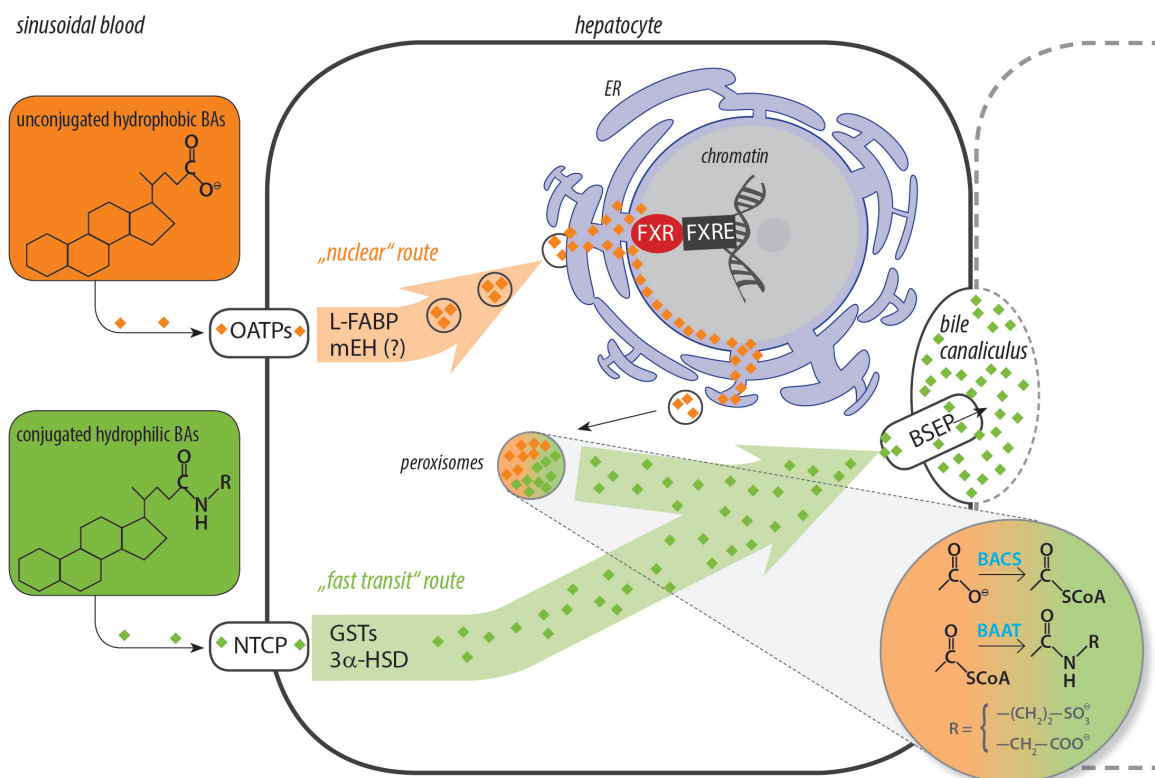
This apparent discrepancy between the high liver exposure of T-OCA (but also of the parent OCA itself) and the weak effect on liver target genes suggests that in rodents, at least, OCA acts as an intestinally biased FXR agonist. Very few truly liver-specific FXR transcriptional activity markers are known that can be determined from plasma or serum samples without the need for a liver biopsy; histidine-rich glycoprotein (HRG) is one such, although it has the disadvantage of showing only small (although very reproducible) changes over time [82]. It would be interesting to see if plasma HRG levels change upon OCA administration in humans.

In the few publications where synthetic FXR agonists have been compared with OCA in animal models, potent examples such as WAY-450 and PX20606 demonstrate more extensive FXR-mediated effects than OCA at comparable doses. Hambruch et al. compared PX20606 with OCA and demonstrated that, despite similar potency in biochemical and cellular reporter assays, PX20606 was more potent than OCA in every *in vivo* model tested, from high fat diet-induced dyslipidemia in wild type, ApoE<sup>-/-</sup> or LDLR<sup>-/-</sup> mice to normolipidemic monkeys [41].

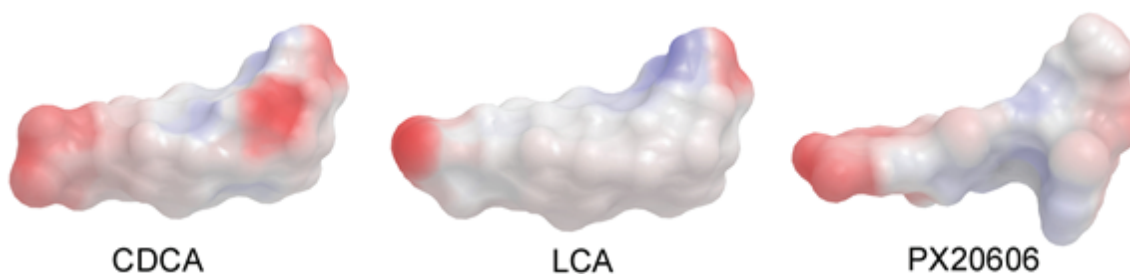
Why is this? The clear implication is that the highly conjugated pool of OCA is not transcriptionally active in the liver, due to “fast lane” trafficking through the hepatocytes. The low percentage of unconjugated OCA might account for the limited liver transcriptional activity observed, with the great proportion of *in vivo* activity attributable instead to intestinal agonism. Synthetic FXR ligands such as PX20606 or GW4064 bear terminal carboxylic acids but are otherwise quite lipophilic. Their properties are therefore reminiscent of unconjugated hydrophobic BAs such as DCA or LCA (see Figure 3 for Connolly surface polarity plots of PX20606 in comparison to LCA), and it is reasonable to assume that their intracellular hepatic disposition (and hepatic transcriptional potential) is also similar.

Synthetic FXR agonists such as PX20350 and PX20606, both synthesized in our labs, also form the taurine amide *in vivo*. The conjugates are not significantly orally bioavailable (FdP <10%, unpublished observations), and this prevents their enterohepatic circulation. In rodents the tauro conjugate of PX20606 represents up to 40% of the total compound pool in the liver. While a significant proportion, this is considerably lower than for OCA and the residual unconjugated drug levels are still high. Nonetheless, it remains unclear whether for this compound the tauro conjugate does contribute to the very strong transcriptional effects observed.

Experimentally, cell culture systems are limited in their capacity to reflect the physiological considerations described above. Data addressing the capacity of various BAs to elicit FXR transcriptional responses in either immortalized cell lines or primary hepatocytes are conflicting. Consistent with our proposed model, Vaquero et al. reported that cell lines that lack FXR become sensitive to FXR transcriptional activation by unconjugated but not by conjugated BAs



**Figure 2:** Model of proposed routes for transhepatic flux of different types of bile acids: Conjugated hydrophilic BAs seem to take the “fast transit” (green) route through the hepatocyte without “touching” FXR to a larger extent whereas unconjugated, hydrophobic BAs tend to take a route which involves binding to L-FABP and transfer to the perinuclear ER membrane from where they are delivered to FXR (orange route). Abbreviations: OATPs (= organic anion transport polypeptides), NTCP (= sodium-dependent bile acid cotransporting polypeptide), L-FABP (=liver-specific bile acid binding protein), mEH (=microsomal epoxide hydrolase), GSTs (= Glutathione-S-transferases), 3 $\alpha$ -HSD (= 3-alpha hydroxysteroid dehydrogenase), BSEP (=bile salt export pump), BACS (=bile acyl-CoA synthetase), BAAT (bile-acyl-CoA: amino acid N-Acyltransferase).



**Figure 3:** Electrostatic properties for CDCA, PX20606 and LCA (calculated by the REBEL method in ICM 3.8 from MolSoft L.L.C.) mapped onto their Connolly surfaces. (reddish: partial or full negative charge, blueish: partial positive charge, white: hydrophobic area).

upon transfection of FXR cDNA. But in the same study, primary hepatocytes or cell lines with endogenous FXR expression were similarly sensitive to FXR activation by both conjugated and unconjugated BAs [84]. The authors suggest the presence and differential sensitivity of the four different FXR isoforms (FXR $\alpha$ 1 or 2, with or without a 12bp insert), as one potentially confounding factor; FXR $\alpha$ 1

isoforms have been mainly detected in the liver, with FXR $\alpha$  mainly in the intestine and the kidney [85]. It is difficult, however, to test different FXR agonists for their potency at different FXR isoforms since the outcome is likely biased by the type of promoter/reporter construct and the cellular environment that is used to determine transcriptional activity levels. Biochemical assays are also not suitable for testing

different isoforms, since they typically employ the ligand binding domain only that is common to all FXR isoforms.

In summary, the model that we propose assumes that potent synthetic FXR ligands are bound in the liver to membranes or bile acid binding proteins which normally translocate unconjugated, hydrophobic BAs and present them to FXR. Only when ligands are delivered in this way is FXR activated for transcription. In contrast, ligands that are subject to the Ntcp-dependent conjugated BA transit route are never presented to FXR during hepatic transit and remain transcriptionally silent. It should be clearly stated here, however, that this concept is still speculative and not directly proven. There is only circumstantial evidence from the literature on bile acids and limited experimental observations from synthetic FXR agonists that fit into our proposed model of two different transhepatic routes, one coupled to FXR transcriptional activation and the other one not or to a lesser extent.

## 2. Conclusion: What is Required in a Therapeutically Effective FXR Ligand?

The model proposed above has consequences for the design of potent FXR-targeted drugs.

If both intestinal and liver FXR activation is desired – and to what extent agonist effects are required in either of these organs is still under debate – then a highly amphiphilic FXR ligand bearing a carboxylic acid for transport recognition together with a highly lipophilic body for potent membrane insertion and hence transcriptional FXR activation is desirable. Would FXR agonists be desirable that avoid taurine conjugation but are still effective FXR activators in the liver? Permanent and overly potent FXR activation might be dangerous since FXR is also described as a major driver of hepatic regeneration after liver injury of hepatectomy [86]. Hepatic FXR drives the expression of FoxM1, a transcription factor that orchestrates hepatocellular proliferation [87]. Sustained activation of FXR in the liver might potentially result in liver cell proliferation and ultimately bears the risk of forming neoplasias.

From our experience, the following considerations need to be taken into account in the design of potent FXR agonists *in vivo*, provided their intrinsic affinity to FXR as demonstrated by biochemical FXR assays is sufficient ( $< 100$  nM EC<sub>50</sub>).

1. Amphiphilic structure with a lipophilic body and a protruding terminal moiety bearing a carboxylic acid. An acid isostere might yield similar *in vivo* potency and may prevent formation of acyl glucuronides but may lead to uncontrolled drug accumulation in the liver. It is not unambiguously proven whether the presence of an acidic function is mandatory for potent liver transcriptional activity *in vivo* as an element of direct FXR binding, or whether its role is in locating potent

FXR agonists in ER and nuclear membranes, or in binding to a chaperone protein such as L-FABP.

2. Plasma concentrations are not predictive of the potency of FXR agonists *in vivo*, and liver concentrations similarly do not correlate directly with activity. The potential of a drug for hepatic FXR activity can only be addressed through gene expression analysis (applicable only in laboratory animals) or the secretion of liver specific markers (e.g., HRG in primates and humans).
3. As a pharmacodynamic marker for intestinal FXR activation, FGF15/FGF19 concentration in plasma over time can serve a valuable role. Many other markers of bile acid synthesis (e.g., 7- $\alpha$ -hydroxy-4-cholesten-3-one, “C4”) are not useful for differentiating intestinal from liver FXR activity since they derive from changes in the activity of Cyp7A1, which is under control of the intestinally driven FXR-FGF19-FGFR4 axis as well as of hepatic FXR itself.
4. If a potent liver transcriptional response is observed by a candidate FXR ligand it is important to establish that this is not attributable to liver accumulation over time. We have mostly observed this phenomenon for overly lipophilic candidates.

In essence, the hunt for a high quality FXR drug candidate involves a delicate balance between potent *in vivo* activity on the one hand and sufficient solubility, bioavailability and lack of liver accumulation or off target effects on the other. What remains as a take home message for a good FXR drug is still:

Give me an “A”, give me an acid (-ic function).

## Competing Interests

The authors declare that they have no competing interests.

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