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Novel bile acid derivatives as promising therapeutic approach for liver and metabolic disorders

Doctoral thesis

Hradec Králové, 2021 Mgr. Alžbeta Štefela

I hereby declare that this thesis is my original work which I solely composed by myself under the supervision of Prof. PharmDr. Petr Pávek, Ph.D. All used literature and other sources are summarized in the list of references and properly cited. This work has not been submitted for any different or equal degree.

Prohlašuji, že tato práce je mým původním autorským dílem, které jsem vypracovala samostatně pod vedením svého školitele prof. PharmDr. Petra Pávka, Ph.D. Veškerá literatura a další zdroje, z nichž jsem při zpracování čerpal, jsou uvedeny v seznamu použité literatury a v práci řádně citovány. Práce nebyla využita k získání jiného nebo stejného titulu.

……

Mgr. Alžbeta Štefela

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ABSTRACT IN ENGLISH LANGUAGE

Candidate: Mgr. Alžbeta Štefela

Supervisor: Prof. PharmDr. Petr Pávek, PhD.

Title of the doctoral thesis: Novel bile acid derivatives as promising therapeutic approach

Bile acids (BAs) are amphipathic steroidal molecules that are ~~traditionally~~ known to facilitate intestinal digestion and absorption of lipids and fat-soluble substances. On top, the recent findings have revealed that they represent important signaling agents involved in the orchestration of lipid, glucose and energy metabolism and immune response. BAs exhibit these roles by activating intracellular nuclear receptors such as farnesoid X (FXR), pregnane X (PXR) vitamin D receptors. Furthermore, BAs act as endocrine signaling molecules and activate numerous biological cascades via a membrane G-protein-coupled receptor, termed TGR5. Therefore, the extensive modulation of BA scaffold underwent to identify compounds with specific targeting of above-mentioned receptors as a promising therapeutic approach for the treatment of various liver and metabolic disorders including cholestasis, biliary cirrhosis, nonalcoholic steatohepatitis or diabetes.

The principal aim of this doctoral thesis was to investigate the structure activity relationship (SAR) between bile acid-derived ligands and receptors involved in bile acid signaling. To address this goal, we used ~~a~~ complex approach combining *in vitro* and cell-free assays with molecular docking. Selected derivatives were then investigated in different cellular models as well as *in vivo* in mice. We demonstrated that acetyl derivatives of deoxycholic and cholic acid are PXR ligands. In the next study, we described 3β-isoobeticholic acid as a low-affinity FXR ligand, which readily epimerases to obeticholic acid in hepatic cells and therefore become a strong FXR agonist in cellular and animal models. In addition, we determined 3,7-dehydroobeticholic acid as a potent TGR5 ligand with minimal activity toward FXR. Then, we introduced a novel bile acid derivative with unique, first-in-class, combined FXR antagonistic and TGR5

agonistic activity. The compound had no off-target activation and represented the most efficient TGR5 agonist among steroidal compounds described, so far.

The second aim of this doctoral thesis was to evaluate anti-inflammatory capacity of ursodeoxycholic acid derivatives in human THP-1-derived macrophages. We showed that a derivative termed UDCA-18:1LPE suppressed a release of inflammatory cytokines by the inhibiting the recruitment of adaptor proteins into lipid rafts which led to an attenuated activation of p38, JNK, and NF-κB signaling pathways. These results highlighted the previously observed protection by UDCA-18:1LPE *in vivo*.

In conclusion, the studies elaborated in this dissertation contributed to the understanding of SAR between compounds derived from bile acids and their receptors with the description of the downstream signaling and effects.

ABSTRAKT V ČESKÉM JAZYCE

Kandidát: Mgr. Alžbeta Štefela

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Název disertační práce: Nové deriváty žlučových kyselin jako slibný terapeutický přístup jaterních a metabolických onemocnění

Žlučové kyseliny (ŽK) jsou amfipatické steroidní molekuly, které, jak je obecně známo, usnadňují trávení a vstřebávání lipidů a látek rozpustných v tucích. Kromě toho, nedávný výzkum ukázal, že ŽK představují důležité signalizační molekuly zapojené do řízení lipidového, glukózového a energetického metabolismu a imunitní odpovědi. ŽK vykazují tyto role aktivací intracelulárních jaderných receptorů, jako jsou receptory farnesoidní X (FXR), pregnanový X (PXR) anebo vitaminový D receptory. Navíc, ŽK fungují jako endokrinní signální molekuly a aktivují řadu biologických kaskád prostřednictvím membránového receptoru spojeného s G-proteinem, nazývaného jako TGR5. V důsledku těchto zjištění, struktura ŽK byla extenzívně modifikována s cílem identifikovat sloučeniny se specifickým zaměřením na výše zmíněné receptory jako slibný terapeutický přístup k léčbě různých jaterních a metabolických poruch včetně cholestázy, biliární cirhózy, nealkoholické steatohepatitidy nebo cukrovky.

Hlavním cílem této disertační práce bylo zkoumat vztah struktury a aktivity (SAR) mezi ligandy odvozenými od žlučových kyselin a receptory zapojenými do jejich signalizace. K dosažení tohoto cíle jsme použili komplexní přístup kombinující *in vitro* a *in silico* nebuněčné testy včetně molekulárního dokování. Vybrané deriváty byly poté zkoumány na různých buněčných i *in vivo* myších modelech. Ukázali jsme, že acetylderiváty deoxycholové a cholové kyseliny jsou ligandy PXR. V další studii jsme popsali kyselinu 3β-isoobeticholovou jako ligand FXR s nízkou afinitou, ale snadno epimeizující v jaterních buňkách na kyselinu obeticholovou, čímž vzniká silný agonista FXR. Kromě toho jsme identifikovali kyselinu 3,7-dehydroobeticholovou jako silný ligand TGR5 s minimální aktivitou na FXR. V následné studii jsme představili

nový derivát žlučových kyselin s unikátní kombinovanou FXR antagonistickou a TGR5 agonistickou aktivitou. Tenhle derivát působil specificky na receptorech ŽK a představoval nejúčinnější agonistu TGR5 z dosud popsaných steroidních sloučenin.

Druhým cílem této disertační práce bylo vyhodnotit protizánětlivou kapacitu derivátů kyseliny ursodeoxycholové na lidských makrofázích odvozených od THP-1. Ukázali jsme, že derivát označovaný jako UDCA-18:1LPE potlačuje uvolňování zánětlivých cytokinů inhibicí přesunu adaptačních proteinů do lipidových raftů, což následně vedlo k snížené aktivaci signálních drah p38, JNK a NF-kB. Tyto výsledky zdůraznily dříve pozorovanou protekci pomocí UDCA-18:1LPE *in vivo*.

Závěrem, studie vypracované v této disertační práci přispěly k pochopení SAR mezi sloučeninami odvozenými od žlučových kyselin a jejich receptory s popisem následné signalizace a účinků.

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1. INTRODUCTION

Bile acids are steroid molecules with amphipathic properties, synthetized exclusively by hepatocytes (primary bile acids) and further converted merely by microbial enzymatic activity in the distal ileum and colon into secondary bile acids. Along with their roles in emulsification and absorption of lipids and fat-soluble substances, research effort of many laboratories over the last two decades revealed the significance of bile acids as the important signaling molecules linked to the regulation of various metabolic processes and inflammation [1].

In addition, bile acids represent the key agents in cholesterol turnover. They facilitate its absorption and importantly, bile acids by themselves are the terminal products of cholesterol catabolism representing the major pathway of cholesterol elimination from the body [2]. Although the fractional loss of bile acid is relatively small, it is compensated by the daily *de novo* synthesis from cholesterol amounting up to 500 mg in adult humans [3]. This accounts for approximately 50% of total cholesterol disposal in the body [4].

1.1. Traditional view of bile acids

De novo synthesis of bile acids from cholesterol is a complex process, demanding synchronization of 17 distinct enzymes in various compartments of hepatocytes including endoplasmic reticulum, cytosol or peroxisomes [5]. The cholesterol can be transformed to bile acids via two different pathways: the classic "neutral" or the alternative "acidic" pathway (Figure 1). At the end of this multistep enzymatic reaction, water-insoluble cholesterol is converted into amphipathic molecules – primary bile acids chenodeoxycholic (CDCA; 3α,7α,12α-dihydroxy-5β-cholan-24-oic acid) or cholic acid (CA; 3α,12α-dihydroxy-5β-cholan-24-oic acid).

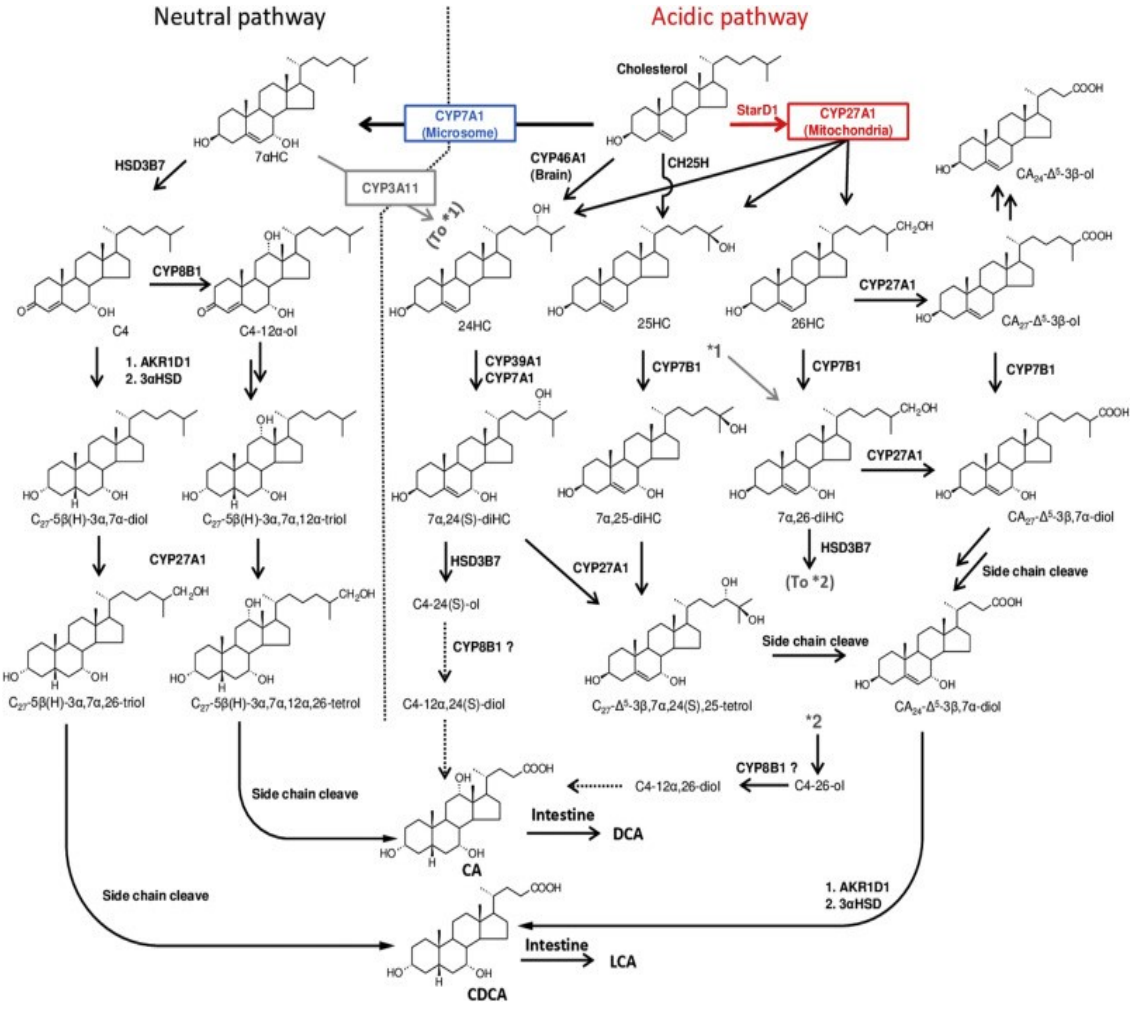

***Figure 1**. De novo synthesis of bile acids from cholesterol in mammalian hepatocytes [6]*

Synthetized bile acids are immediately conjugated by the bile acid coenzyme A synthetase (BACS) and N-acetyltranferase (BAT) in the peroxisomes. These enzymes are tremendously effective since more than 98% of bile acids excreted from the liver are conjugated, usually with glycine or taurine [7-9]. In humans, the proportion of glycine to taurine conjugates depends solely on the disposition of these amino acids and probably does not have any biological impact [10]. The conjugation significantly enhances hydrophilicity of the molecules and thereby increases their detergent capacity. This decreases their pKa end ensures almost complete ionization at physiological pH. At this point, bile acids conjugates can be also termed as bile salts [11]. Since the negative charge makes them impermeable to the cell membranes, they need to be actively secreted through the canalicular membrane of hepatocytes by efflux

transporters. Subsequently, they are carried by bile ducts to the gallbladder where they are stored during a fasting period representing the core part of the bile [12, 13].

Food ingestion stimulates the release of cholecystokinin from duodenum which in turn promotes gallbladder contractions resulting in a bile flow and delivering the bile to the intestine. During this process bile acids in high millimolar concentration reach critical micellar concentration and therefore they allow the solubilization of dietary lipids. The emulsified fat is accessible for pancreatic lipase to be broken down to monoacylglycerides and fatty acids [14]. The concentration of bile acids remains high in duodenum, jejunum and proximal ileum, where along the digestion, bile salts facilitate the absorption of lipid soluble substances including hormones, vitamins and cholesterol. Pursuing the intestinal passage, bile acids are absorbed through enterocytes and rich portal circulation. Their passage through both, apical and sinusoidal membranes, is fully dependent on transporters activities which are the most effective in the distal ileum. From enterocytes, bile acids are secreted to the blood and associated with plasma proteins and re-uptaken by transporters on the sinusoidal membrane of liver cells. The whole cycle including excretions of bile acids into the bile, their intestinal passage, reabsorption and return into the liver via portal vein is called the enterohepatic circulation (Figure 2) [2, 15].

The ileal transporters of bile acids are very efficient, recapturing around 90-95% of bile acid conjugates. The escaped bile acids (accounting approximately 400-800 mg/day in adult healthy human) undergo robust bacterial transformation in the colon. Large intestine harbors a complex microbial flora which directly influences the bile acid composition [11] by generating secondary bile acids: deoxycholic acid (DCA; 3α,12α-dihydroxy-5β-cholan-24-oic acid) and lithocholic acid (LCA; 3α-hydroxy-5β-cholan-24-oic acid), ursodeoxycholic acid (UDCA; 3α,7β,12α-dihydroxy-5β-cholan-24-oic acid) and other minor species. Bacterial density in the human colon is the highest find in the nature, accounting 10^12 bacteria per 1 gram of wet weight of feces [16, 17]. The bacterial modifications in distal ileum and colon comprise deconjugation catalyzed by

bacterial hydroxylases, oxidation of hydroxyl groups at C3, C7, C12 positions and 7α/β dehydroxylation which rise hydrophobicity and pKa of these bile acids enabling their recovery via passive absorption across the colonic epithelium [11, 18]. Nevertheless, hydrolysis and dihydroxylation are associated with increased toxicity linked to the pathogenesis of cholesterol gallstone disease and colon cancer [19]. The relation between bile acids and intestinal bacteria is reciprocal and rather complex. While the intestinal microbiome transforms bile acids and therefore modulates their physiochemical and biological properties, bile acids possessing detergent properties and antimicrobial activities regulate bacterial colonization [20]. Failure of this mechanisms, e.g. during biliary obstruction, lead to the bacterial overgrowth which may result in malabsorption, gut epithelial injury and bacterial translocation [21]. Consistently, an increase in abundance of bile acids in the colon causes inhibition of bacterial growth and result in the alteration of microbiome [22].

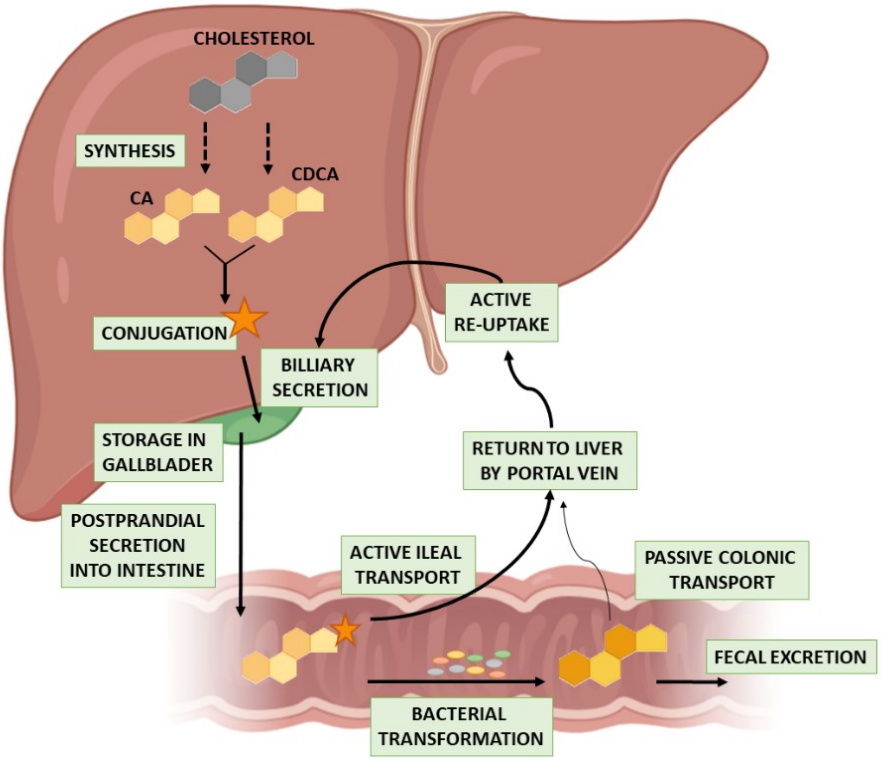

Figure 2. *Enterohepatic circulation of bile acids. Light yellow steroidal scaffolds represent primary bile acids, darker yellow steroidal scaffold represent secondary bile acids formed by intestinal bacteria. The orange star indicates conjugation of bile acid with glycine or taurine.*

2. RECEPTORS ACTIVATED BY BILE ACIDS

Discoveries from last two decades have elucidated the role of bile acids as important signaling molecules that can be considered as hormones orchestrating bile acid, lipid and glucose homeostasis or inflammation. They exhibit these effects not only due to their physiochemical properties but mainly by activation of multiple membrane and nuclear receptors collectively known as bile acid receptors (BARs)[23-25].

2.1. Farnesoid X receptor

Farnesoid X receptor (FXR) is a ligand-activated transcription factor identified in 1995 and named after its first-recognized activator farnesol, an intermediate product in the biosynthesis of cholesterol [26, 27]. Four years later, FXR was declared as the bile acid receptor (BAR) after three groups independently reported that FXR is activated by primary bile acids [28-30]. Up to date, there are two identified FXR genes, FXRα (NR1H4 – nuclear receptor classification: nuclear receptor subfamily 1, group H, member 4) and FXRβ (NR1H5) in mammals. The latter one is considered as a pseudogene in primates and humans [31, 32] whereas FXRα gene encodes four isoforms arising from a transcription starting at distinct promoters and an alternative splicing of the mRNA [33, 34]. Although all isoforms share identical ligands and spectrum of the downstream genes, their different tissue expression and sensitivity to target genes have been described [35, 36].

2.1.1. FXR - Mechanism of action

Upon activation, FXR binds to the specific nucleotide sequences on the promoter sequences of target genes known as FXR response elements (FXREs), which are composed of two inverted repeats (AGGTCA) with one nucleotide spacing (IR-1) [37]. The interaction of FXR with DNA results in transcriptional up-regulation or repression of the target genes but also might not initiate any

particular action [38-40]. FXR can interact with FXREs as a monomer but it binds preferentially in a permissive heterodimeric complex with retinoid X receptor α (RXRα, NR2B1), so it can be activated by ligands of both partners (bile acids and/or retinoic acid) [41, 42]. In the resting state, FXR is associated with co-repressors, such as SMRT/NCOR [43], that are dissociated upon conformational changes of FXR induced by a ligand binding followed by recruitment of co-activators [44]. Although many co-activators have been described (SRC-1, PRMT-1, CARM-1, GPC2, PGC (PPAR-g coactivator)-1a, ASCOM, TRRAP, p3000, SIRT1, DRIP-205, KU proteins and others [45-54], their significance in the regulation of specific genes remains to be elucidated [55].

2.1.2. FXR - Structure

Consistently to other nuclear receptors, FXR consists of a highly conserved DNA-binding domain in the N-terminal region with characteristic two cysteine-rich zinc finger motifs involved in DNA binding and dimerization. The ligand-binding domain (LBD) is situated in the C-terminal region. The three-dimensional structure of the FXR LBD was resolved with different steroidal or non-steroidal ligands by various groups providing detailed insights on the LBD structure and ligand binding. However, a missing co-crystallization with natural ligands, native receptor or full-length receptor limits the interpretation of the proposed structures. The FXR LBD is composed of 12 α-helices (H1-12) adjacent in three anti-parallel layers in a so-called "α-helical sandwich" (Figure 3) [56]. This alignment forms a lipophilic ligand binding pocket which is able to accommodate small lipophilic molecules. The activation function 2 (AF2) corresponding to H12 is a multifarious and functionally essential part of the LBD.

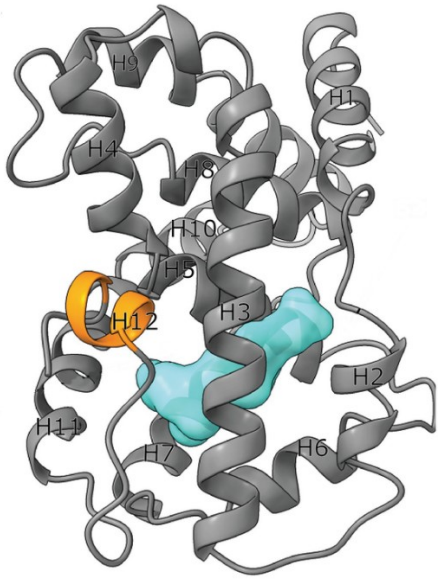

***Figure 3**. 3D representation of the farnesoid X receptor ligand binding domain (FXR LBD). The ligand binding pocket is indicated in light azure color, activation function -2 (AF-2) located in the helix 12 (H12) is orange (modified from [56])*

Upon ligand binding, AF2 undergoes conformational changes closing the ligand binding pocket, coordinating of co-repressors and co-activators association and dimerization [57]. Besides bile acids, FXR LBD recognizes a spectrum of endogenous compounds including oxysterol-22(R)-hydroxy cholesterol, androsterone, poly-unsaturated fatty acid such as arachidonic acid or docosahexaenoic acid [58-62]. However, bile acids possess the highest affinity to FXR among all natural ligands, being about 1000-times more potent than other cholesterol metabolites or steroid hormones. This is explained by the specific orientation of the bile acid scaffold caused by A/B ring juncture in the *cis* conformation ensuing deflection of the A ring from planar orientation (Figure 4). As a consequence, the bend A ring stabilizes the AF2 in the active position by indirect interactions with triad residues W466 (H12), H444 (H11), and Y358 (H10), respectively (the numbering corresponds to 1OSV crystal) [41, 57, 63, 64]. In addition, the carboxylic tail of bile acids is oriented to the posterior section of the ligand binding pocket whereas other steroidal compounds acquire inverted position facing their oxidized tails to the AF2 located close to the entry of the pocket.

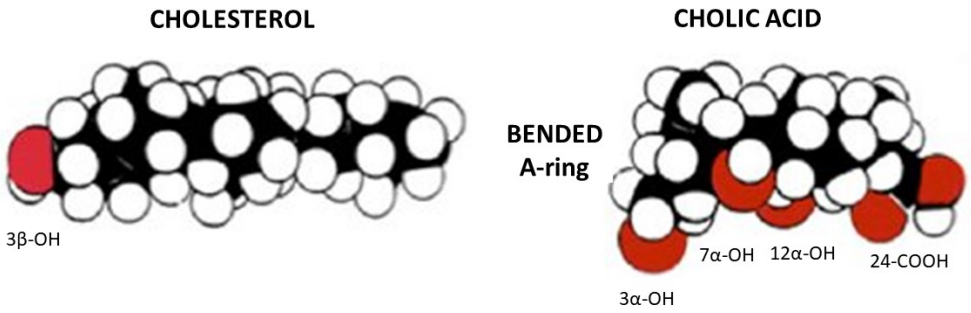

Figure 4. *Space-filling models of cholesterol and cholic acid (modified from [25])*

2.1.3. FXR – Ligands and SAR of bile acids

Bile acids binds FXR with different affinities, with CDCA being the most potent endogenous ligand followed by DCA, CA and LCA. The relative affinities of bile acids for FXR are determined by the orientation axial hydroxyl groups at the C7 and C12 positions whilst the conjugation has little impact on the binding (Figure 5) [41, 57, 63, 65].

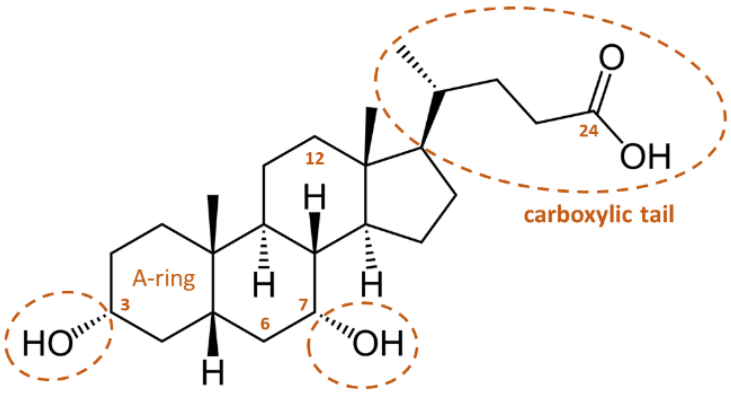

Figure 5. Chenodeoxycholic acid

Intensive structural modifications of bile acids scaffold in recent years provided important knowledge of structure activity relationship (SAR):

- Introduction of an alkyl group to the 6α position increases dramatically affinity to FXR by fitting the remaining free space in the FXR ligand binding pocket. Indeed, 6α-ethylchenodeoxycholic acid (6-ECDCA, obeticholic acid, OCA) is the most potent steroidal FXR agonist known so far with EC_{50} in nanomolar concentration [66].

- The hydroxyl group at 7α position is essential for the affinity to FXR and it is considered as pharmacophoric. Indeed, its epimerization to 7β position leads to complete abrogation of the activity toward FXR, e.g. 7β epimer of CDCA, UDCA, does not activate FXR [65, 67-70].
- The orientation of the hydroxyl group at the position C3 has a smaller significance on direct FXR activation but it is rather important for stabilization of the interactions with FXR cavity [68, 69, 71].
- Structural changes on the carboxylic tail including reduction to alcohol, substitution by sulfate group, modification of the length or conjugation has little impact on FXR activation [69, 72].

Except bile acids and their derivatives, FXR is activated by nonsteroidal ligands. Recently, a great progress has been made in the discovery of numerous nonsteroidal agonists for FXR such as GW4064, AGN29, AGN31 or WAY-362450 (FXR 450) [73, 74]. GW4064 is the most frequently described FXR ligand but it exhibits cytotoxicity and limited bioavailability that have restricted its further use [75, 76].

2.1.4. FXR – tissue expression

FXR is abundantly expressed in the tissues orchestrating bile acid homeostasis and enterohepatic circulation such as the liver and small intestine with the highest occurrence in the ileum [77]. In the liver, FXR is dominantly expressed in the parenchymal cells and to lower extent in the endothelial, stellate and Kupffer cells [78, 79]. In addition, FXR was detected in the organs that are not primarily targeted by bile acids including kidney, adrenal glands, immune cells, stomach, lungs, heart, ovary thymus, eye, spleen, skin, testes or brain [80-83]. This wide expression suggests that FXR might be engaged in different physiological roles in many organs.

2.1.5. FXR-biological roles

2.1.5.1. FXR regulation of bile acid homeostasis and enterohepatic circulation

The rate-limiting enzyme in the synthesis of bile acid from cholesterol, 7α-hydroxylase, CYP7A1, is regulated by negative bile acid-dependent feedback loop at the transcriptional level [84]. However, FXR does not bind directly to the putative bile acid response element (BARE) in its promoter but it controls CYP7A1 gene expression via two distinct mechanisms. First, it up-regulates expression of the atypical nuclear receptor small heterodimer partner (SHP, NR0B2) in the hepatocytes which in turn promotes dissociation of coactivators of the hepatic nuclear factor 4 (HNF4, NR2A1) or the liver receptor homolog-1 (LRH-1 NR5A2) [85, 86]. HNF4 and LRH-1 bind to the BARE region of the CYP7A1 gene and transactivate its expression [24, 41]. Secondly, FXR induces secretion of fibroblast growth factor -19 (FGF-19, corresponding to murine FGF-15) from enterocytes. FGF-19 via activation of the hepatic FGF receptor 4 (FGFR4) and represses CYP7A1 as well as CYP8B1 genes expression by interfering with the c-Jun N-terminal kinase pathway in hepatocytes [87, 88].

Increased accumulation of relatively hydrophobic bile acids promotes cellular injury in the liver parenchyma. The activation of FXR triggers protective mechanisms which rise hydrophilicity of bile acids. FXR positively regulates enzymes involved in bile acid conjugation: the bile acid coenzyme A synthetase (BACS) and N-acetyltranferase (BAT) [89]. Conjugated bile acids are actively excreted into bile by the bile salt export pump (BSEP, ABCB11) across the canalicular membrane. Hepatocyte-specific expression and high substrate specificity for conjugated bile acids for BSEP has been reported. BSEP expression is strongly induced by FXR at the transcriptional level [90-92]. FXR mediates inhibition of the Na^+-taurocholate co-transporting peptide (NTCP, SLC10A1), the major basolateral transporter of bile acids [93]. This represents another mechanism by which FXR protects hepatocytes against bile acid accumulation.

Additionally, FXR stimulates oxidation, sulfatation and glucuronidation of bile acids by up-regulating CYP3A4 [94], dehydroepiandrosterone-sulfotransferase (SULT2A1) [38] and uridine glycuronosyltransferase 2B4 (UGT2B4) enzymes [40], respectively. Sulfated and glucuronidated bile acids are excreted into the bile via multidrug resistance-associated protein 2 (MRP2, ABCC2) which is also positively regulated by FXR [95].

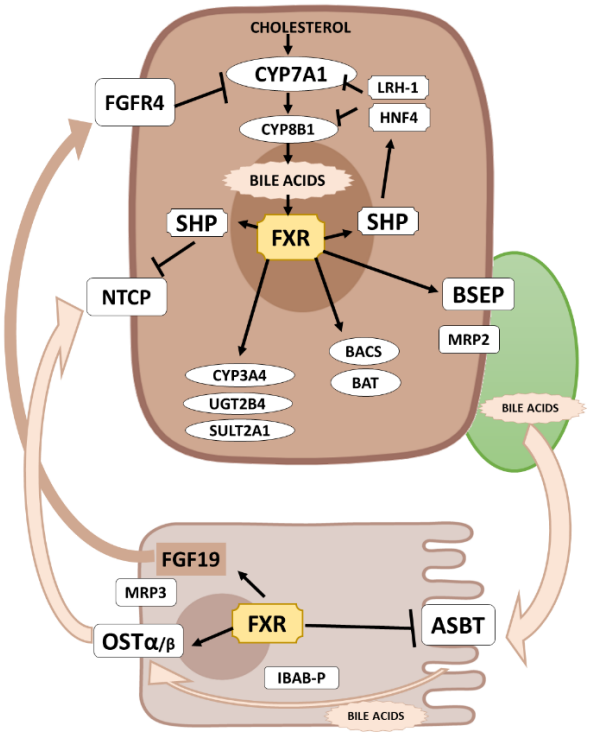

Figure 6. *Overview of the mechanisms by which FXR regulates bile acid homeostasis and enterohepatic circulation. In hepatocytes, FXR negatively regulates bile acids synthesis by inhibiting CYP7A1, the rate-limiting enzyme in cholesterol conversion. After activation, FXR induces SHP expression, which in turn interacts with HNF4 and LRH-1 to suppress transcription of CYP7A1. Simultaneously, FXR induces FGF19 which down-regulates CYP7A1 via FGF4 receptor. FXR induces expression of conjugation enzymes BACS and BAT and detoxification enzymes CYP3A4, UGT2B4 and SULT2A1. FXR activates expression of bile acid export transporters BSEP and MRP2 while inhibits bile acid import by negative regulation of NTCP. In enterocytes, FXR suppresses up-take of bile acids from intestinal lumen by inhibiting ASBT transporter activity and increases OSTα/β expression to elevate bile acid excretion to portal vein.*

The bile acid pool in the body is maintained relatively constant. In addition to the regulation of bile acid synthesis, this is ensured by regulation of reabsorption of bile acids and their transport across enterocytes during enterohepatic circulation. For this purpose, FXR has been described to induce intestinal bile acid binding protein (I-BABP) which facilitates the transport of bile acids across

enterocytes. FXR also stimulates excretion of bile acids from enterocytes into the portal circulation via the organic solute transporters OSTα (SLC51A) and OSTβ (SLC51B) and inhibits the apical sodium-dependent like bile salt transporter (ASBT, SLC10A2) involved in the uptake of bile acid conjugates from intestinal lumen into enterocytes (Figure 6) [81, 96, 97].

2.1.5.2. FXR roles in lipid homeostasis

FXR regulates lipid and lipoprotein metabolism by altering the transcription of several genes involved in fatty acid and triglyceride synthesis and lipoprotein secretion and plasma clearance. FXR down-regulates sterol response element binding protein-1c (SREBP-1c) in the liver via SHP-dependent manner [98]. SREBP-1c represents the master gene regulator of a wide range of genes participating in fatty acid and triglyceride synthesis such as acetyl-CoA carboxylase (ACC), acetyl-CoA synthetase (AceCS) and fatty acid synthetase (FASN). Moreover, FXR stimulates activity of lipoprotein lipase via induction of its activator, apolipoprotein (apo)C-II [99], and inhibition of its repressor, apoC-III [100]. By these mechanisms, FXR activation enables to lower plasma level of triglycerides and very low-density lipoprotein (VLDL)[101]. FXR was also reported to regulate some other genes involved in lipid metabolism such as fatty acid transporter CD36, peroxisome proliferator-activated receptors (PPARs) or syndecan-1 [102, 103]. Of interest, FXR KO mice exhibit proatherogenic lipoprotein profile with increased triglycerides and cholesterol plasma levels along with circulating free fatty acids. However, FXR deficiency prevented from formation of atherosclerotic lesions in mice on high fat diet [49] suggesting rather intricate involvement of FXR signaling in the regulation of lipoprotein homeostasis.

2.1.5.3. FXR roles in sugar homeostasis

The role of FXR in glucose metabolism is complex. FXR KO mice exhibit elevated plasma glucose levels and impaired insulin sensitivity [104]. FXR ligands

have been shown to lower expression of enzymes involved in gluconeogenesis including phosphoenol-pyruvate carboxylase (PEPCK) and glucose -6-phosphatase (G6Pase)[105]. On the other hand, high levels of glucose attenuate FXR expression implying mutual regulation between FXR and plasma glucose [106]. Of note, the effect on glucose homeostasis mediated by FXR might not be beneficial during obesity [107].

2.1.5.4. FXR roles in inflammation

In addition to its roles in metabolic regulation, FXR is an interesting therapeutic target of inflammatory processes in various diseases. FXR acts as a negative regulator of nuclear factor kappa B (NF-κB) pathway which is the master mediator of inflammatory response [108]. In addition, activation of FXR has been shown to suppress inflammatory cell infiltration and liver inflammation in the murine model of nonalcoholic steatohepatitis (NASH) [109, 110], inhibited secretion of inflammatory cytokines and chemokines such as TNFα, IL-1β, IL-6 [111, 112], and suppressed inducible nitric oxide synthetase (iNOS) and cyclooxygenase 2 (COX-2) [113].

2.2. Other nuclear receptors sensed by bile acid

Besides FXR, bile acids interact with pregnane X receptor (PXR), vitamin D receptor (VDR) and constitutive androstane receptor (CAR).

PXR is mainly expressed in the gastrointestinal tract and liver, where it represents a xenobiotic sensor inducing phase I and II metabolism genes [114]. In hepatocytes, PXR is activated by cytotoxic LCA and promotes its detoxification via induction of CYP3A4 and SULT2A1 [95]. In addition, PXR agonists inhibit synthesis of bile acids from cholesterol by down-regulating CYP7A1 expression via direct interaction with HNF4 in SHP-independent manner [115].

VDR is mainly engaged in the mineral and bone homeostasis and cellular differentiation. VDR is broadly expressed in various tissues including intestine,

kidney, pancreas, adipose tissues [116]. Interestingly, VDR is expressed in human, but not in murine hepatocytes [78, 117]. Similarly to PXR, VDR is activated only by LCA (among endogenous bile acids) and involved in the regulation of CYP7A1 and CYP3A4 [117, 118].

CAR is expressed in the liver and gastrointestinal tract and plays an important role in detoxification by up-regulation of CYP2B monooxygenases or sulfotransferases [119, 120]. CAR has been shown to be activated by a bile acid metabolite 6-keto-LCA [121].

2.3. Takeda G-protein coupled receptor

Takeda G-protein coupled receptor (TGR5, also referred as G-protein coupled bile acid receptor 1, GP-BAR1, or membrane-bile acid receptor, M-BAR) is the most described membrane receptor activated by bile acids [122-124]. Although GP-BAR1 is the official designation declared by the NIH gene database, TGR5 name remains to be preferred by the scientific community and I will also use this abbreviation in this work (Figure7).

2.3.1. TGR5 mechanism of action

TGR5 is a cell-surface receptor and a member of the class A of G-protein coupled receptors (GPCRs). In 2002, three years after the identification of FXR as the nuclear receptor for bile acids, TGR5 was reported to be almost exclusively activated by bile acids [122]. However, the crystal structure of TGR5 receptor was introduced only very recently, in 2020 [125]. Upon ligand binding, conformational changes on the receptor allow the release of the G-protein complex consisting of α, β and γ subunits. Subsequently, GDP is replaced by GTP which promotes the dissociation of G-protein-α subunit. This activates generation of cAMP to further affect intracellular signaling via protein kinases A and its downstream signaling [123].

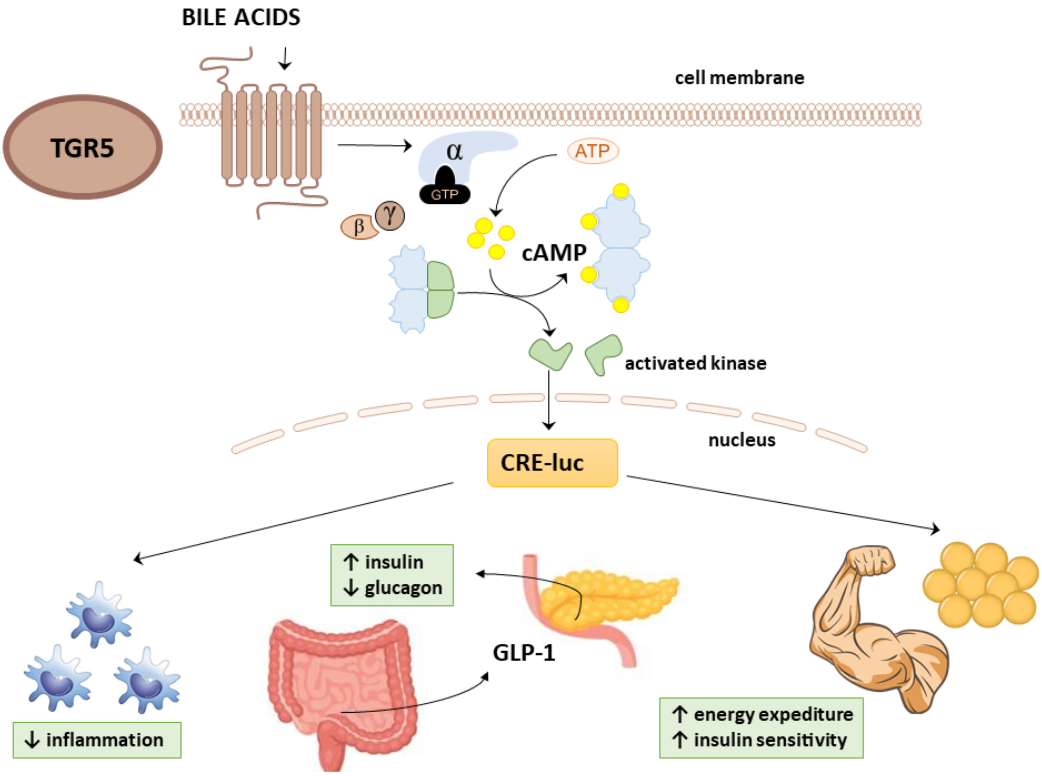

Figure 7. *TGR5 is G-protein coupled receptor which upon activation results in cAMP production which further activate kinases that stimulate cAMP response elements (CRE) in promoters of target genes involved in various effects in different organs. TGR5 activation inhibits cytokine production in macrophages. Bile acids activate TGR5 in enteroendocrine cells an thus stimulate the release of GLP-1 which in turn increases insulin and reduces glucagon secretion. TGR5 activation in skeletal muscle and adipose tissue increases energy expenditure and insulin sensitivity.*

2.3.2. TGR5 ligands

TGR5 is activated by both conjugated and unconjugated BAs in dose-dependent manner. Taurine conjugate of litocholic acid (TLCA) is the most potent natural agonist followed by LCA > DCA > CA and CDCA [123]. Several other compounds found in the nature were identified as weak TGR5 activators such as oleanolic acid extracted from olive leaves [126] or obacunone found in citrus fruit [127].

The modulation of bile acid scaffold led the discovery of more selective and potent TGR5 ligands. First, the methylation of C-23(S) position was described to shift the activating capacity toward TGR5 over FXR [128]. Further investigations led to the discovery of 6α-ethyl-23(S)-methyl-cholic acid (S-EMCA or INT-777), the most potent and selective TGR5 agonist described so far [129]. Additional

studies on the steroidal scaffold proposed the modification of hydroxyl group at C3 or C7 position to increase activity on TGR5 [130].

Additionally, TGR5 can be activated with many diverse synthetic compounds such as carboxamide or nicotinamide derivatives [131].

2.3.3. TGR5 tissue expression

TGR5 is widely distributed in various tissues and organs including gallbladder, intestine, stomach, pancreas, spleen, heart, skin, or adipose tissue and muscles [132]. Interestingly TGR5 is densely expressed in the submucosal nerve plexus of the small and large intestine [133]. In the liver, it is not found on the membrane of hepatocytes, but it is broadly localized on Kupffer cells and liver sinusoidal endothelial cells [134, 135].

2.3.4. Biological roles of TGR5

2.3.4.1. Body weight control

Recent study showed that BA administration to mice fed with a high fat diet prevented weight gain of animals by increased energy expenditure mediated in murine brown adipose tissue and human skeletal muscle [136]. This effect was attributed to TGR5/cAMP dependent activation of the iodothytonine deiodinase, the enzyme converting inactive thyroxine into triiodothyronine, the active intracellular mediator of basal metabolism [137]. This effect was mediated by the TGR5 signaling and was independent of FXR. Interestingly, the treatment with FXR agonist GW4064, increased fat accumulation in adipose tissue in mice [138]. Within consideration of liver diseases, preliminary studies with TGR5 agonists suggest that activation of TGR5 may attenuate lipid loading and impairment of liver function by restoring levels of liver enzymes in murine models. However, the precise mechanism remains to be elucidated [139, 140].

2.3.4.2. Glucose metabolism

The beneficial effects of circulating bile acids on glucose homeostasis have been reported (Patti ME 2009) and linked to TGR5 activation. Bile acids temper glucose homeostasis via TGR5 induced glucagon like peptide 1 (GLP-1) secretion from enteroendocrine cells [141-143] that results in improvement of insulin sensitivity. In addition, it was reported that TGR5 is expressed in pancreatic β-cells where it mediates insulin secretion [144].

2.3.4.3. Inflammation

TGR5 is densely expressed on the membranes of monocytes and macrophages and acts as negative modulator of inflammatory response. Its activation attenuates production of pro-inflammatory cytokines from lipopolysaccharide or TNFα activated macrophages and therefore abrogates propagations of inflammation through the cAMP–NF-κB-dependent pathway [123, 145, 146]. This was further confirmed by various studies on TGR5-transfected macrophages as well as on alveolar macrophages where BAs were shown to diminish their phagocytic activity. Moreover, studies with selective TGR5 agonist, INT-777, proved that TGR5 activation protects from adipose tissue inflammation via AKT-mTOR downstream signaling [147]. Other studies using INT-777 amended anti-inflammatory potential of TGR5 by diminishing intraplaque inflammation in murine model of atherosclerosis. In addition, TGR5 signaling decreased LDL cholesterol particles uptake in macrophages underlying its antiatherogenic effects [148].

2.4. Other membrane receptors sensed by bile acids

Besides TGR5, bile acids have been reported to interact with sphingosine 1-phosphate receptor 2 (SIPR2) in hepatocytes [149]. SIPR2 upon activation by conjugated bile acids triggers ERK1/2 and AKT signaling [150] which is considered to stabilize SHP and therefore inhibit CYP7A1 signaling. Therefore, it

is involved in regulation of lipid and glucose homeostasis [50, 135]. Secondary bile acids DCA and LCA activates muscarinic receptor M_2 and M_3 in central nervous system and smooth muscle cells [151] whereas CDCA antagonizes formyl peptide receptor 1 (FMLP) in macrophages [152]. To note, the role of CDCA on vascular endothelial growth factor (VEGF-R) has been described in cancer cell lines [153].

3. TARGETING BILE ACID NETWORK IN THE DRUG DEVELOPMENT

Therapeutic perspectives of bile acids originated by the observation of Nobel prize laureate Philip Hench in 1938 who remarked that manifestation of rheumatic arthritis diminished when patients become jaundiced [154]. In folk Chinese medicine, the bile from black bears was used to cure hepatobiliary diseases for many centuries. Its main component is ursodeoxycholic acid (UDCA) which is nowadays the number one drug in the treatment of gallstones and cholestasis [155]. Today, a plethora of compounds derived from bile acids, or targeting their signaling pathways is being developed and investigated for the treatment of liver, inflammatory and metabolic diseases.

3.1. UDCA and derivatives

UDCA is produced by bacterial epimerization of 7α hydroxyl group of CDCA in the intestine and represents small proportion of bile acid pool in humans (1-3%) [156, 157]. However, the orientation of the hydroxyl group to β position strongly increases hydrophilicity of UDCA and is associated with choleretic and hepatoprotective properties. Although UDCA is not an FXR ligand, it exhibits a lot of actions resembling *bona fide* FXR activation. For example, maintenance of bile acid homeostasis, anti-inflammatory and anti-fibrotic effects, and improvement of serum lipids. On the other hand, UDCA exhibited also FXR-antagonizing behavior on lipid metabolism [158], however, if UDCA is an FXR antagonist

remain to be elucidated. UDCA is used for the treatment of gallstones, primary biliary cirrhosis (PBC) and primary sclerosing cholangitis (PCS). Beneficial effects on fatty liver disease or NASH were reported to be rather limited [159].

To improve its characteristic and pharmacokinetic behavior, several derivatives have been proposed. The synthetic side-chain-shortened UDCA analogue 24-nor-UDCA is supposed to be resistant to amidation, which enables its cholehepatic shunting. Recently, norUDCA has been successfully tested clinically in patients with PSC [160].

3.2. FXR agonist OCA

The introduction of an ethyl moiety at position C6 on the B-ring of CDCA led to the discovery of OCA, one of the most potent steroidal FXR agonists [66]. Until preparation of this work, OCA has been introduced together in 27 clinical trials (14 have been completed yet) as monotherapy or in combinational therapy with other drugs (https://clinicaltrials.gov, 30.07.2020). The majority of clinical trials focused on therapeutic effects of OCA in patients with primary biliary cholangitis (PBC).

PBC is an autoimmune chronic liver disease characterized by progressive non-suppurative destruction of small bile ducts leading in cholestasis, hepatic injury and ultimately liver cirrhosis. Addition of OCA to the therapy has resulted in decreased level of alkaline phosphatase and decreased or stabilized levels of bilirubin in two phase II studies [161, 162] and one Phase III study [163, 164] (all of them were double blinded and randomized) when administrated alone or together with UDCA. Moreover, OCA has shown to reduce liver injury biomarkers (alanine aminotransferase, aspartate aminotransferase and gamma-glutamyl transferase) levels as well as inflammatory markers including tumor necrosis factor α, C-reactive protein or Immunoglobulin M. The most dominant adverse effects accompanying treatment with OCA were pruritus, nausea, and fatigue. No serious adverse effects have been related to OCA treatment. OCA was approved by FDA in 2016 for treatment of PBC in combination with UDCA in adults with an

inadequate response to UDCA, or as monotherapy in adults unable to tolerate UDCA. Currently, safety and efficacy of OCA is being evaluated in two ongoing studies – COBALT (https://clinicaltrials.gov/ct2/show/NCT02308111) and OCARELIFE (https://clinicaltrials.gov/ct2/show/NCT03703076).

Non-alcoholic fatty liver disease (NAFLD) and NASH are currently the most frequent chronic liver disorders with increasing prevalence worldwide characterized by lipid accumulation in the liver associated with increased hepatic inflammation that may eventually lead to cirrhosis, hepatocellular carcinoma or liver-related mortality. In the FLINT trial (NCT01265498), administration of OCA for 72 weeks to NASH patients improved hepatic steatosis and inflammation, hepatocellular ballooning and fibrosis. In addition, OCA reduced liver enzymes – ALT, AST, GST, but increased ALP levels. Moreover, OCA worsened cholesterol concentration (increased LDL and decreased HDL cholesterol) and exacerbate insulin resistance. OCA treatment was not sufficient to resolute NASH [165, 166]. Insulin sensitivity plays a crucial role in the development of NASH and OCA administration to patient with NAFLD and diabetes 2 type resulted in increased insulin sensitivity and lowered hepatic inflammation and fibrosis [167]. In another clinical trial (CONTROL, NCT02633956), OCA has shown superior effect to lipid lowering drug atorvastatin. When co-administrated together, OCA treatment resulted in more significant reduction of LDL concertation, particle size and particle concentration comparing to atorvastatin treatment alone in NASH patients [168].

Furthermore, beneficial effects of OCA have been investigated in clinical trials for therapy of primary sclerosing cholangitis (AESOP, NCT02177136), alcoholic hepatitis (TREAT, NCT02039219), gallstone disease, bile acid diarrhea [169], lipodystrophy or obesity [170].

3.3. TGR5 activation

Considering TGR5 activation governs a wide spectrum of metabolic and inflammatory regulated processes, therapeutic targeting of TGR5 is proposed as

a novel approach to cure disorders linked to inflammation or impaired metabolism. However, none of the TGR5 activators have been examined in clinical trials, yet [171].

3.4. FGF19 mimetics

FGF15/19 induced by FXR activation has been reported to reduce lipid accumulation in the liver and ameliorate insulin sensitivity [172, 173]. However, FGF19 signaling has been also associated with development of hepatocellular carcinoma [174, 175] which arised concerns for its potential therapeutic targeting. FGF19 analogue NGM282 exhibited no cancerogenic effect but improved hepatic steatosis and fibrosis in patients with NASH [176, 177]. The most abundant side effects from the therapy was hypercholesterolemia, but recently it has been shown that this could be sufficiently overcome by addition of statin to the therapy [178].

3.5. ASBT inhibitor

Absorption of conjugated bile acids in the distal ileum is mediated via ASBT transporter (or SLC10A2) [179]. Intestine-specific ASBT-inhibitor were developed as novel approach for type-2 diabetes based on the idea that accumulated bile acid in the intestine will increase TGR5 activation in enteroendocrine L-cells leading to enhanced GLP-1 secretion. Indeed, this strategy reduced level of plasma glucose in animal models and human diabetic patients [180-182]. In addition, ASBT inhibitor alleviated hepatic steatosis and inflammation [183], increased hepatic bile acid synthesis and decreased plasma cholesterol and exhibited liver protective effects [184-186]. Even though intestinal ASBT inhibition brought multiple beneficial effects in various clinical trial for treatment of type 2 diabetes (T2D) and NASH, the treatment is associated with bile acid diarrhea caused by high concentration of bile in the colon that stimulates chloride and water secretion into the lumen which may be serious limitation in the therapy.

3.6. FXR antagonists

Despite encouraging results from clinical studies, a therapy with FXR agonists have shown multiple obstacles including contradictory outcomes and arising side effects such as altered cholesterol plasma levels, cholestasis or constipation [187]. This favors for the development of FXR antagonists as potential target for dyslipidemia or cholestatic disorders [188]. Unfortunately, the development of FXR antagonists has not been prioritized in past years and the knowledge about structure and activity relationship is limited.

Of interest, first substance with described FXR antagonistic properties was guggulsterol isolated from the tree *Commiphora mukul* traditionally used in ayurvedic medicine as lipid-lowering agent [189]. However, later examinations have reported that guggulsterol is a promiscuous agent interacting with a spectrum of nuclear receptors [190-192]. More selective FXR antagonistic characteristics were described in the sterols isolated from marine organisms such as suvanine [193] or sulfated sterols theonellasterol and their derivatives [194]. Furthermore, theonellasterol prevented liver damage induced by bile duct ligation [195].

Consistently with FXR agonists, some non-steroidal compounds were described to antagonize FXR. Substituted pyrazolone derivatives decreased total cholesterol and triglycerides *in vitro* and in mice fed with cholesterol-rich diet [196]. N-acetylated piperidine derivatives suppressed lipid accumulation in differentiated adipocytes [197] and benzoic acid derivative exhibited antidyslipidemic properties by increasing HDL and decreasing non-HDL cholesterol and triglycerides in hamster model of dyslipidemia [198]. Another FXR antagonist, termed SIPI-7623 decreased triglycerides and cholesterol levels in HepG2 cells and ameliorated diet-induced atherosclerosis in rat and rabbit models [199].

The antagonistic impact on FXR signaling was reported to positively regulate glucose levels in *db/db* mice [200]. HS218, a selective FXR antagonist, suppressed gluconeogenesis in primary murine hepatocytes and improved glucose

homeostasis in various models of diabetes in mice [201]. The beneficial effect of FXR antagonists on glucose homeostasis is additionally supported by the findings that metformin, a drug of choice for T2D, inhibits intestinal FXR signaling [202].

3.7. Bile acids sequestrants

Bile acid sequestrants are well-known and safe hypolipidemic agents. From the chemical perspective, they are polymeric resins that act as ion-exchangers for bile acids and therefore prevent their absorption from the ileum. By this action, they stimulate *de novo* synthesis of bile acid from cholesterol in a feed-back manner and thereby increase the cholesterol uptake from blood into the liver [203]. In addition to the treatment of dyslipidemia, bile acid sequestrants are successfully used in the therapy of diarrhea caused by bile acid malabsorption which occurs e.g. during Crohn disease [204]. Recently, they have been shown to have beneficial effects in NASH by attenuation of inflammatory infiltration [205] and modulation of glycemia [206]. In past, patients suffering from severe itching caused by accumulated bile acids were treated with bile acid sequestrants [207].

3.8. Modulation of intestinal microbiome

Bile acids are the key agents for cholesterol elimination. Increased bile acid deconjugation by bacterial hydrolysis in the distal ileum limits their effective active uptake resulting in increased excretion of bile acid in feces [208]. Based on this principle, administration of probiotics has been proposed to reduce cholesterol levels by increasing its clearance [209]. In addition, modulation of bile acid microbiota by probiotics reduced liver steatosis and inflammation in patient with fatty liver disease [210, 211]. On the other hand, the suppression of bacterial growth decreases their potential to oxidize bile acid scaffold. The treatment with antibiotics or antioxidants led to increased levels of muricholic acid conjugates, the endogenous FXR antagonists in mice. The altered bile acid composition protected against diet induced obesity and alleviated steatosis [185, 212].

4. AIMS OF THE WORK

The aims of this work are:

1. Study structure activity relationships (SAR) between compounds derived from bile acid and receptors involved in bile acid signaling including direct interaction with nuclear receptor LBDs, investigation of their effects in cellular models and *in vivo* in mice
2. Investigate anti-inflammatory potential of the ursodeoxycholic acid derivatives in human THP-1-derived macrophages

5. LIST OF PUBLICATION RELATED TO THE DOCTORAL THESIS TOPIC

The aims of this doctoral thesis were accomplished and published in following publications in international peer-reviewed journals with impact factor (on the older publications, I have my maiden name Horvatova):

1. **Stefela A**, Kaspar M, Drastik M, Holas O, Hroch M, Smutny T, Skoda J, Hutnikova M, Pandey AV, Micuda S, Kudova E, Pavek P **(2020)** 3β-Isoobeticholic acid efficiently activates the farnesoid X receptor (FXR) due to its epimerization to 3α-epimer by hepatic metabolism. *J Steroid Biochem Mol Biol*. 202:105702 (IF 2019 = **3.813, Q2**)

2. **Horvatova A**, Utaipan T, Otto AC, Zhang Y, Gan-Schreier H, Pavek P, Pathil A, Stremmel W and Chamulitrat W **(2018)** Ursodeoxycholyl lysophosphatidylethanolamide negatively regulates TLR-mediated lipopolysaccharide response in human THP-1-derived macrophages. *European journal of pharmacology* 825:63-74. (IF 2018 = **3.170, Q2**)

3. Carazo A, Hyrsova L, Dusek J, Chodounska H, **Horvatova A,** Berka K, Bazgier V, Gan-Schreier H, Chamulitrat W, Kudova E and Pavek P **(2017)** Acetylated deoxycholic (DCA) and cholic (CA) acids are potent ligands of pregnane X (PXR) receptor. *Toxicol lett* 265:86-96 (IF 2017 = **3.166, Q2**)

4. Publication submitted to Journal of Medicinal Chemistry (IF 2019 = **6.205, Q1**) in March 2021: Kaspar M,# **Stefela A,#** Drastik M, Kronenberger T, Micuda S, Dracinsky M, Klepetarova B, Pavek P, Kudova E **(2021)** (E)-3α-Hydroxy-7-ethylidene-5β-cholan-24-oic acid is the highly potent steroidal dual G-protein bile acid receptor 1 (GPBAR1) agonist/farnesoid X receptor (FXR) antagonist.
   # authors contributed equally

6. OTHER PUBLICATIONS NOT RELATED TO THE DOCTORAL THESIS TOPIC

1. Boltnarova B, Kubackova J, Skoda J, **Stefela A**, Smekalova M, Svacinova P, Pavkova I, Dittrich M, Scherman D, Zbytovska J, Pavek P, Holas O **(2021)** Plga based nanospheres as a potent macrophage-specific drug delivery systém. *Nanomaterials*. 11:749 (IF 2019 = **4.324, Q2**)

2. Skoda J, Dusek J, Drastik M, **Stefela A**, Dohnalova K, Chalupsky K, Smutny T, Micuda S, Gerbal-Chaloin S, Pavek P **(2020)** Diazepam Promotes Translocation of Human Constitutive Androstane Receptor (CAR) via Direct Interaction with the Ligand-Binding Domain. *Cells*, 9(12), 2532 (IF 2019 = **4.366, Q2**)

3. Smutny, T., Dusek, J., Hyrsova, L., Nekvindova, J., **Horvatova, A**., Micuda, S., Gerbal-Chaloin, S., Pavek, P. **(2020)**. The 3'-untranslated region contributes to the pregnane X receptor (PXR) expression down-regulation by PXR ligands and up-regulation by glucocorticoids. *Acta pharmaceutica Sinica. B*, *10*(1), 136–152 (IF 2019 = **7.097, Q1**)

4. Dusek J., Skoda J., Holas O., **Horvatova A.**, Smutny T., Linhartova L., Hirsova P., Kucera O., Micuda S., Braeuning A., Pavek P. **(2019)** Stilbene compound trans-3,4,5,4´-tetramethoxystilbene, a potential anticancer drug, regulates constitutive androstane receptor (Car) target genes, but does not possess proliferative activity in mouse liver. *Toxicol lett* 313:1-10 (IF 2019 = **3.569, Q1**)

7. AUTHOR'S CONTRIBUTION

In this section, I specify my contribution to these manuscripts.

1. In the manuscript published in The Journal of Steroid Biochemistry and Molecular Biology, I performed following:
    - the design of experiments with the supervision of Prof. PharmDr. Petr Pavek Ph.D.
    - cell culture of HepG2, Huh-7 and HepaRG cells including their differentiation and treatment
    - reporter gene assays with *wt* and mutant expression vectors, RT-qPCR, sample preparation for LC-MS and Western blotting
    - analysis of obtained data from all experiments and preparation of all Figures, except Figure 1
    - preparation of a draft of the manuscript and participated on the revisions of publication

2. In my first manuscript published in European Journal of Pharmacology, I performed following experiments:
    - cell culture of the THP-1 monocytic cell line, their differentiation to macrophages and treatment
    - ELISA experiments for Figure 1D-F, all Western blot analysis, immunofluorescence experiments including the quantification of the nuclear translocation, lipid rafts isolation and lipid extraction
    - analysis of obtained data and preparation of the Figures
    - preparation of a draft of the manuscript

3. In this manuscript published in Toxicology Letters, I performed:
    - cell cultivation and lipid extraction for HPLC-MS analysis

4. In this paper submitted in Journal of Medicinal Chemistry I did:
    - experiment design with the supervision of Prof. PharmDr. Petr Pavek Ph.D.
    - cell culture of HepG2, Huh-7, AML12, NCI-H716 and HepaRG cells including their differentiation and treatment
    - reporter gene and FRET assays, RT-qPCR analyses, measurement of cAMP production and GLP-1 secretion, cytotoxicity assays
    - data analysis from all experiments and prepared Figures 2, 4, 5, 6, 7, and 8.
    - I wrote a draft of the manuscript.

8. COMMENTARY ON THE PUBLISHED PAPERS IN INTERNATIONAL JOURNALS WITH IMPACT FACTORS RELATED TO THE DOCTORAL THESIS TOPIC.

1. **Stefela A**, Kaspar M, Drastik M, Holas O, Hroch M, Smutny T, Skoda J, Hutnikova M, Pandey AV, Micuda S, Kudova E, Pavek P **(2020)** 3β-Isoobeticholic acid efficiently activates the farnesoid X receptor (FXR) due to its epimerization to 3α-epimer by hepatic metabolism. *J Steroid Biochem Mol Biol*. 202:105702 (IF 2019 = **3.813, Q2**)

In this paper, we introduced obeticholic acid (OCA) derivatives that where synthetized based on the idea that OCA undergoes bacterial biotransformation to these putative compounds in the intestine. Therefore, we presented C3 and C7 epimers, or oxidized forms of OCA and aimed to study their activities on nuclear receptors targeted by bile acids including farnesoid X, pregnane X, vitamin D and constitutive androstane receptors (FXR, PXR, VDR, CAR) as well as the membrane GPBAR1 receptor.

For this purpose, we performed multiple luciferase reporter gene assays of above-mentioned receptors and we found out that 3β epimer of OCA activates FXR to the same extent as does OCA itself in HepG2 cells. But when we examined their capacities to recruit an FXR coactivator protein SRC-2 to the FXR ligand binding domain in a cell free assay, we observed a 9-times lower effect of the 3β-hydroxyl epimer. To resolve the discrepancy, we hypothesized that 3β-isoOCA might be converted to OCA in cells. Therefore, we subjected treated cells and cell culture media samples to LC-MS analysis. Indeed, we observed OCA formation in samples treated with 3β-isoOCA, however, no 3β-isoOCA was observed in OCA-treated samples suggesting one-way conversion. Subsequently, we identified 3β-hydroxysteroid dehydrogenase and aldo-ketoreductase, enzymes involved in the conversion of cholesterol to bile acids, to catalyze the conversion of 3β-isoOCA. The epimerization was suppressed in the presence of inhibitors of these enzymes, trilostane and bromsalicylic acid, respectively Finally, we did not observe any difference in the FXR downstream genes expression in HepG2, Huh7 and

terminally differentiated cells on mRNA and protein levels after OCA and 3β-isoOCA treatments. Therefore, we concluded that 3β-isoOCA represents a pro-drug of OCA activated in hepatocytes.

Our secondary aim was to investigate the activity of the introduced compounds on the membrane G-protein coupled bile acid receptor (GP-BAR1, TGR5). Interestingly, we discovered that oxidation of C3 and C7 hydroxyl groups (3,7-dehydroOCA) lead to a strong decrease in affinity to FXR but significantly increased affinity to GPBAR1. These results were brought by our molecular docking analysis. Since the crystal structure of GP-BAR1 has not been published so far, the precise 3D model of GPBAR1 is constructed based on the amino acid sequences and shared structural characteristics within the family of G-protein coupled receptors. However, the knowledge of binging modes of bile acids and their derivatives is limited and differ in different models. To resolve this, we prepared mutants of GPBAR1 in predicted key amino acid residues and compared the affinity of tested compounds on wild type and mutated GPBAR1 receptors. We concluded that Ser270 is crucial for binding and activation of GPBAR1 whereas the Glu169 residue participates on stabilization of the activated receptor by tested bile acid compounds.

2. **Horvatova A**, Utaipan T, Otto AC, Zhang Y, Gan-Schreier H, Pavek P, Pathil A, Stremmel W and Chamulitrat W **(2018)** Ursodeoxycholyl lysophosphatidylethanolamide negatively regulates TLR-mediated lipopolysaccharide response in human THP-1-derived macrophages. *European journal of pharmacology* 825:63-74. (IF 2018 = **3.170, Q2**)

The aim of this work was to investigate anti-inflammatory potential of ursodeoxycholyl lysophophatidylethanolamide (UDCA-LPE). UDCA-LPE is a bile acid-phospholipid conjugate that have previously been shown to protect from liver injury in murine models of hepatic damage. We hypothesized that its hepatoprotective properties might result from a direct targeting of immune cells.

For this purpose, we chose human the THP-1 monocytic cell line, since they can be differentiated by phorbol ester to macrophages representing liver reside macrophages – Kupffer cells. Subsequently we stimulated them with bacterial lipopolysaccharide (LPS) and we assessed the effect of UDCA-LPE on the inflammatory response. Performing ELISA, we observed that the release of pro-inflammatory cytokines TNF-α, IL-6 and IL-1β was suppressed when THP-1-derived macrophages were pre-treated with UDCA-LPE. Additionally, we assessed UDCA-LPE derivatives with different fatty acid moiety incorporated and we found that an oleyl-UDCA-LPE was the most potent one in the suppression of cytokine production. Moreover, UDCA-LPE exhibited also anti-migration activity on THP-1 derived macrophages.

Next, we aimed to investigate the involvement of various inflammatory signaling cascades at molecular level. By Westerm blot analysis, we determined that UDCA-LPE reduced phosphorylation of p38 and JNK1/2. In addition, the bile acid-phospholipid conjugate inhibited the translocation of NF-κB, an important mediator of inflammation. Upon LPS activation of Toll-like receptor 4 (TLR4), myeloid differentiation factor 88 (Myd88) and TNF associated factor 6 (TRAF6) adaptor proteins are recruited to the activator complex in the lipid raft and can trigger activation of above-mentioned cascades. We found that UDCA-LPE reduced the LPS-stimulated recruitment of Myd88 and TRAF6 into the lipid rafts.

Finally, UDCA-LPE restored the LPS caused decrease of protective polyunsaturated fatty acid (PUFA) species in THP-1 macrophages.

We can conclude that UDCA-LPE showed specific anti-inflammatory effects in human THP-1-derived macrophages. The underlying mechanism involved an inhibition of LPS-activated recruitment of adaptor proteins into lipid rafts leading to suppression of p38, JNK1/2 and NF-κB signaling that finally results in reduced secretion of inflammatory cytokines. Moreover, UDCA-LPE inhibited migration of macrophages and rescued the loss of LPS-induced loss of PUFA. Therefore, UDCA-LPE might be suitable for further development of drugs for treatment of inflammatory diseases.

3. Carazo A, Hyrsova L, Dusek J, Chodounska H, **Horvatova A,** Berka K, Bazgier V, Gan-Schreier H, Chamulitrat W, Kudova E and Pavek P **(2017)** Acetylated deoxycholic (DCA) and cholic (CA) acids are potent ligands of pregnane X (PXR) receptor. *Toxicol lett* 265:86-96 (IF 2017 = **3.166, Q2**)

In this study, we assessed the ability of acetylated bile acid derivatives to interact with FXR, PXR, VDR and CAR. For this purpose, we transiently co-transfected HepG2 cells with appropriate luciferase expression vectors and nuclear receptor constructs and assessed the effects of newly synthetized acetylated derivatives on them.

We found out, that acetylation at C3, C7 and C12 positions lead to the decrease of affinity to FXR but might increase the affinity to PXR. Indeed DCA 3,12-diacetate and CA 3,7,12-triacetate. Were found to be potent PXR activators. Accordingly, we observed high affinity to PXR ligand binding domain determined by TR-FRET using a recombinant PXR assay as well as by molecular docking analysis. None of the compounds significantly activated VDR and CAR. PXR is known to up-regulate expression of CYP3A4 and CYPAB6 in order to increase the detoxification capacity of the liver. We observed increased CYP3A4 and CYP2B6 mRNA levels in differentiated HepaRG cells after treatment with DCA 3,12-diacetate.

Finally, we analyzed human bile and mouse liver samples for the presence of acetylated bile acid derivatives employing HPLC-MS analysis. However, all the samples were negative for presence of DCA 3,12-diacetate and CA 3,7,12-triacetate, suggesting these compounds are not endogenous ligands for PXR.

4. Publication submitted to Journal of Medicinal Chemistry (IF 2019 = **6.205, Q1**) in March 2021: Kaspar M,# **Stefela A,#** Drastik M, Kronenberger T, Micuda S, Dracinsky M, Klepetarova B, Pavek P, Kudova E **(2021)** (E)-3α-Hydroxy-7-ethylidene-5β-cholan-24-oic acid is the highly potent steroidal dual G-protein bile acid receptor 1 (GPBAR1) agonist/farnesoid X receptor (FXR) antagonist.
   # authors contributed equally

In our most recent manuscript, we introduced a family of 7-alkylated derivatives of chenodeoxycholic acid, the most potent endogenous FXR ligand. We found that the alkylation at the position C7 led to the abrogation of capacity to activate FXR. Nevertheless, the compounds exhibited a significant capacity to antagonize FXR activated by synthetic or natural ligands in gene reporter assays. We concluded that FXR antagonizing behavior depended on the length and the saturation of the alkyl chain. Subsequent analysis using a cell-free coactivator recruitment assays confirmed the FXR antagonizing capacity. To further unravel the antagonistic mechanism and interactions within the FXR ligand binding pocket, we performed molecular dynamics simulations that suggested competitive mechanism of action since both FXR agonist and antagonist shared similar orientation.

The biggest highlight of this manuscript was the discovery of (E)-3α-hydroxy-7-ethylidene-5β-cholan-24-oic acid (compound 2a) as a molecule endowed with unique, first-in-class activity towards BA receptors. This includes FXR antagonizing capacity (IC_{50} = 14 μM). In addition, we showed that compound 2a antagonized FXR signaling in primary human hepatocytes and differentiated hepatic HepaRG cells at mRNA as well as protein level. Interestingly, compound 2a did not interact with any other nuclear receptor but showed to be a potent TGR5 ligand (EC_{50} = 26 nM). Such a combined targeting of FXR and TGR5 has been proposed for the therapy of diseases with disturbed glucose homeostasis as we demonstrated with increased secretion of glucagon-like peptide 1 from human intestinal cells.

9. DISCUSSION

The end products of cholesterol catabolism, bile acids, are well known to facilitate digestion of dietary fat and fat-soluble substances. The recent studies have shown that bile acids are also important signaling molecules orchestrating bile acid, glucose and lipid homeostasis and inflammation acting via nuclear and membrane receptors [1]. These findings accelerated investigation of bile acids and their derivativities with ameliorated characteristics and led to the discovery of obeticholic acid (OCA) as a first steroidal and high-affinity FXR ligand in therapy [66]. Currently, OCA is approved for the treatment of primary biliary cholangitis in UDCA-resistant or intolerant patients and it has been evaluated in various clinical trials for treatment of NASH, T2D or dyslipidemia [170]. Besides its promising results, the treatment of OCA is associated with undesirable side effects such as itching or alteration of cholesterol levels that lead to even deeper investigation of signaling pathway activated by bile acids and their SAR. It has been reported that different FXR ligands might activate different downstream pathways in various organs [89, 213]. Therefore, the development of tissue specific FXR agonist or by selective targeting of FXR downstream genes might overcome side effects associated with bile acid therapy. The detailed description of SAR between bile acid derivatives and are necessary for development of specific FXR modulators.

Bile acids share steroidal scaffold with other steroid molecules, however, the 5β orientation of the hydrogen bend the A ring of the molecule and this unique adjustment have evolved to target FXR [5]. A small modification of bile acid scaffold might change its physiochemical properties and biological characteristics, e.g. epimerization of the hydroxyl group from the position 7α to 7β significantly increases the solubility of the bile acids resulting in lower toxicity [214]. On the other hand, same alteration lead to radical abrogation of the activity on FXR. This was first observed by *Fujino et al.* who compared the activity of CDCA and its 7β-epimer UDCA on FXR [69]. Additionally, they described that reduction of the carboxylic acid to an alcohol does have an omissible impact on FXR activation. Similar conclusion where obtained by *Sepe et al.* who experimented

with 6-ethylcholane scaffold [68]. Accordingly, in our study, 7β-epimer of 3β-isoOCA had no impact on FXR activation whereas 3β-isoOCA preserved strong affinity to FXR. When we profoundly investigated FXR activation by 3β-isoOCA, we observed that it is an equivalent FXR activator as OCA itself in gene reporter assays but exhibit lower affinity to FXR in cell-free TR-FRET assay as well as in molecular docking. By resolving this discrepancy, we found that 3β-isoOCA is efficiently converted to OCA by liver enzymes hydroxydsteroid dehydrogenase and aldo-ketoreductase and represents therefore a hepatocyte-activated pro-drug of OCA. Our findings also support the hypothesis that the hydroxyl group at the C3 position is not crucial for the affinity to FXR but rather help to stabilize FXR LBD in active position [215].

Acetylation decreases hydrophilicity, and PXR is activated by hydrophobic bile acid, such as LCA [216]. Activation of PXR triggers detoxification processes in the hepatocytes by up-regulating CYP3A4 expression docking improved. We described that acetylated DCA and CA are potent ligands of PXR and VDR and with further up-regulation of CYP3A4 mRNA. Accordingly, in our next study, we described acetylation of OCA at the C3 position reduced FXR affinity but increased affinity to VDR [217].

TGR5 activation was associated with itching as an undesirable side effect from bile acid treatment or accumulation [218]. However, TGR5 activation might have a plethora of beneficial impacts including reduced inflammation, increased insulin secretion and sensitivity or improved energy homeostasis. Therefore, TGR5 agonists have been proposed for the treatment of obesity or diabetes [132, 171]. In our study, we discovered 3,7-dehydroOCA being very potent TGR5 activator with limited affinity to FXR. The SAR studies between TGR5 and its ligands were limited by the fact that the crystal structure of TGR5 was unknown at the time of the experimental study. However, several groups proposed detailed models of 3D TGR5 structure sharing many characteristics but differing in binding features and key amino acid residues [219-221]. To overcome this issue, we prepared mutated vector of the key amino acid residues. Consistently with previous findings, we found that interaction between Ser270 and side chain

of BA is inevitable for TGR5 activation. On the other hand, the interaction between Glu169 and C3 hydroxyl group might not be crucial to activate TGR5. This is in accordance with the identification of 3,7-dehydroOCA as potent TGR5 ligand since this bile acid derivative does not have hydroxyl groups to form a hydrogen bond [215].

UDCA-LPE was synthetized as a novel strategy for the treatment of liver injury combining protective characteristics of a phospholipid and UDCA, with the latter one used as a specific carrier to the liver [222]. UDCA-LPE showed good pharmacokinetic profile with effective accumulation in the liver and no hydrolysis of the conjugate were observed. In addition, UDCA-LPE exhibited superior effects to UDCA or PE, or combined UDCA+PE treatment in *in vitro* and *in vivo* models of fulminant liver damage [222-227]. In our published paper, we observed anti-inflammatory capacity of UDCA-LPE in human macrophages, by suppressing production of inflammatory cytokines induced by LPS treatment. The underlying mechanism of protective capacity of this conjugate is rather complex. We observed, that it enables to inhibit LPS-activated phosphorylation of p38, MKK4/7, JNK1/2 as well as translocation of NF-κB in human THP-1 macrophages. We proposed that UDCA-LPE might target the shared upstream TRAF6 by interfering at plasma membrane level. We detected UDCA-LPE in cellular lysates of UDCA-LPE treated THP-1 macrophages suggesting that the conjugate can be taken up, adhered on the cell surface or incorporated into the membrane [228]. Hydrophilic UDCA was described to reside on the interfacial surface however phospholipid species [229] or more hydrophobic bile acid tend to embed into plasma membrane [230]. Unfortunately, we were not able to detect UDCA-LPE by LC/MS-MS analysis due to the presence of detergent in the isolated lipid rafts fractions. By comparing UDCA-LPE with different fatty-acid chains, we observed the most potent inhibitory effects on cytokine production of oleyl and arachidonoyl UDCA-LPE. LPS treatment is associated with increased intracellular LPC containing saturated fatty acids and decrease of polyunsaturated fatty acids (PUFAs). In the study, UDCA-LPE rescued the loss of PUFA-LPC species indicating that it might regulate phospholipid homeostasis. Similar effect has been previously observed in murine hepatocytes and liver, where UDCA-LPE increase

monounsaturated and PUFA containing phospholipids [225, 231]. Our results underlined previously observed hepatoprotective effects of UDCA-LPE and showed that they may arise from anti-inflammatory action not only in epithelial cells but also in macrophages [228].

10. CONCLUSIONS AND FUTURE PLANS

Obesity and related metabolic disorders are prevalent and on the rise in western countries, not omitting the children population. The treatment is, however, often limited to lifestyle modifications which might be effective but difficult for many patients to adhere on. Moreover, the late stages of liver disease are not reversible, lead to the development of hepatocellular carcinoma or liver failure. Therefore, the development of novel therapeutic approach is a high priority.

During my doctoral thesis, I have been working on the research of bile acids, their structure activity relationships and on their complex roles in the regulation of metabolic processes as well as inflammation. The recent research of UDCA derivatives and development of FXR agonists in many laboratories around the world brought a spectrum of compounds with interesting activities. On the other hand, the research of TGR5 pathway or FXR antagonist signaling have been quite neglected in past years, even though it represents an interesting therapeutic approach in many metabolic and inflammatory disorders. Therefore, my future aims are to investigate the involvement of TGR5 and negative FXR regulation in different pathophysiological conditions.

11. CONFERENCES ATTENDED

11.1. ORAL PRESENTATIONS

01/2020 **10th Postgradual and 8th Postdoctorant Scientific Conference, Faculty of Pharmacy, Charles University**
"Discovery of potent GPBAR1 agonists / FXR antagonists"

01/2019 **9th Postgradual and 7th Postdoctorant Scientific Conference, Faculty of Pharmacy, Charles University**
"Novel obeticholic acid keto-derivatives and isomers as potential ligands of bile acid receptors"

01/2018 **8th Postgradual and 6th Postdoctorant Scientific Conference, Faculty of Pharmacy, Charles University**
"Involvement of farnesoid X receptor in novel human cellular model of steatohepatitis"

03/2017 **2nd German Pharm-Tox Summit, Heidelberg, Germany**
"UDCA-LPE alleviates LPS-induced inflammatory response in THP-1-derived human macrophages via suppression of NF-κB and MAPK signaling"

11.2. POSTER PRESENTATIONS

11/2019 **EMBO Practical Course on The fundamentals of high-end cell sorting, Heidelberg, Germany**
"Novel bile acid derivatives as potential therapeutic approach for NASH"

09/2019 **FEBS Advanced Lecture Course on Epigenomics, Nuclear Receptors and Disease, Island of Spetses, Greece**
"Novel obeticholic acid ketoderivatives and isomers as potential ligands of bile acid receptors"

09/2018 **EMBO Workshop on Nuclear receptors and biological networks, Kolymbari, Greece**
"Teriflunomide is an indirect human constitutive androstane receptor (CAR) activator interacting with epidermal growth factor (EGF) signaling"

12. LIST OF ABBREVIATIONS

ACC	Acetyl-CoA carboxylase
AF-2	Activation function 2
AKT	Alpha serine/threonine-protein kinase
ALT	Alanine transaminase
ASBT	Apical sodium–bile acid transporter
ASCOM	ASC-2/NCOA6 complex
AST	Aspartate transaminase
BACS	Bile acid coenzyme A synthetase
BAR	Bile acid receptor
BARE	Bile acid response element
BAT	Bile acid N-acetyltranferase
BSEP	Bile salt export pump
CA	Cholic acid
CAR	Constitutive androstane receptor
CARM1	Coactivator-associated arginine methyltransferase 1
CDCA	Chenodeoxycholic acid
COX2	Cyclooxygenase 2
CYPs	Cytochromes P450
DCA	Deoxycholic acid
DRIP205	Vitamin D-interacting protein 205
ERK	Extracellular signal-regulated protein kinase
FAS	Fatty acid synthase
FGF	Fibroblast growth factor
FMLP	N-Formylmethionyl-leucyl-phenylalanine
FXR	Farnesoid X receptor
FXRE	FXR response element
G6Pase	Glucose 6-phosphatase
GDP	Guanosinediphosphate
GLP-1	Glucagon-like peptide-1

GPBAR-1	G protein-coupled bile acid receptor 1
GPC2	Glypican 2
GPCRs	G-protein-coupled receptors
GTP	Guanosinetriphosphate
HCC	Hepatocellular carcinoma
HDL	High-density lipoprotein
HNF4α	Hepatocyte nuclear factor 4 α
I-BABP	Intestinal bile acid binding protein
IL	Interleukin
iNOS	inducible nitric oxide synthase
IR-1	Inverted repeats
KO	Knockout
LBD	Ligand binding domain
LCA	Litocholic acid
LDL	Low-density lipoprotein
LPS	Lipopolysaccharide
M-BAR	Membrane-type receptor for bile acids
MAPKs	Mitogen-activated protein kinases
MRP2	Multidrug resistance-associated protein 2
NASH	Non-alcoholic steatohepatitis
NF-κB	Nuclear factor kappa B
NRs	Nuclear receptors
NTCP	Na^+-taurocholate cotransporting polypeptide
OATPs	Organic anion transporting polypeptides
OATs	Organic anion transporters
OCA	Obeticholic acid
OST	Organic solute transporter
PBC	Primary biliary cirrhosis
PEPCK	Phosphoenolpyruvate carboxykinase
PGC-1a	PGC (PPAR-γ coactivator)-1a
PPAR	Peroxisome proliferator-activated receptor

PRMT-1	Protein arginine N-methyltransferase 1
PXR	Pregnane X receptor
ROS	Reactive oxygen species
RXRα	Retinoid X receptor α
SHP	Small heterodimer partner
SIPR2	Sphingosine 1-phosphate receptor 2
SIRT1	Sirtuin 1, NAD-dependent deacetylase sirtuin-1
SLC	Solute carrier
SMRT/N-Cor	Silencing mediator for retinoid or thyroid-hormone receptors/Nuclear receptor co-repressor 2
SRC-1	Steroid receptor coactivator-1
SREBP-1c	Sterol regulatory element-binding protein 1
SULTs	Sulfotransferases
T2D	Type 2 diabetes
TGR5	Takeda G-protein coupled receptor
TNF	Tumor necrotizing factor
TR-FRET	Time-resolved fluorescence energy transfer
TRAF6	TNF receptor associated factor 6
UDCA	Ursodeoxycholic acid
UDCA-LPE	Ursodeoxycholyl lysophosphatidylethanolamide
UGTs	UDP-glucuronosyltransferases
VDR	Vitamin D receptor
VEGF	Vascular endothelial growth factor
VLDL	Very-low-density lipoprotein

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14. LIST OF ATTACHMENTS

A1. **Stefela A**, Kaspar M, Drastik M, Holas O, Hroch M, Smutny T, Skoda J, Hutnikova M, Pandey AV, Micuda S, Kudova E, Pavek P **(2020)** 3β-Isoobeticholic acid efficiently activates the farnesoid X receptor (FXR) due to its epimerization to 3α-epimer by hepatic metabolism. *J Steroid Biochem Mol Biol*. 202:105702 (IF 2019 = **3.813, Q2**)

A2. **Horvatova A**, Utaipan T, Otto AC, Zhang Y, Gan-Schreier H, Pavek P, Pathil A, Stremmel W and Chamulitrat W **(2018)** Ursodeoxycholyl lysophosphatidylethanolamide negatively regulates TLR-mediated lipopolysaccharide response in human THP-1-derived macrophages. *European journal of pharmacology* 825:63-74. (IF 2018 = **3.170, Q2**)

A3. Carazo A, Hyrsova L, Dusek J, Chodounska H, **Horvatova A,** Berka K, Bazgier V, Gan-Schreier H, Chamulitrat W, Kudova E and Pavek P **(2017)** Acetylated deoxycholic (DCA) and cholic (CA) acids are potent ligands of pregnane X (PXR) receptor. *Toxicol lett* 265:86-96 (IF 2017 = **3.166, Q2**)

A4. Publication submitted to Journal of Medicinal Chemistry (IF 2019 = **6.205, Q1**) in March 2021: Kaspar M,# **Stefela A,#** Drastik M, Kronenberger T, Micuda S, Dracinsky M, Klepetarova B, Pavek P, Kudova E **(2021)** (E)-3α-Hydroxy-7-ethylidene-5β-cholan-24-oic acid is the highly potent steroidal dual G-protein bile acid receptor 1 (GPBAR1) agonist/farnesoid X receptor (FXR) antagonist.
 # authors contributed equally