Farnesoid X Receptor Agonist for the Treatment of Liver and Metabolic Disorders: Focus on 6-ethyl-CDCA

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Abstract: 6-ethyl-chedeoxycholic acid (6E-CDCA) is a farnesoid X receptor (FXR) ligand endowed with agonistic activity under development for treatment of cholestatic liver diseases including primary biliary cirrhosis (PBC) and liver-related metabolic disorders including non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH). FXR is a bile sensor that acts in coordination with other nuclear receptors to regulate essential steps of bile acid uptake, metabolism and excretion. 6E-CDCA has been investigated in preclinical models of cholestasis, liver fibrosis and diet-induced atherosclerosis. In a phase II clinical trial in patients with PBC, 6E-CDCA met the primary endpoint of a reduction in alkaline phosphatase levels but safety data indicated that the drug exacerbated pruritus, one of the main symptoms of PBC, suggesting that 6E-CDCA or FXR are mediators of pruritus in humans. Treatment of patients with diabetes and liver steatosis resulted in amelioration of insulin sensitivity despite a slight reduction in HDL and increased levels of LDL were observed. These side effects on bile acids and lipid metabolism were all predicted by pre-clinical studies, suggesting that potent FXR ligands hold promise but potential side effects might limit their development.

Keywords: Chenodeoxycholic acid, FXR, MRP4, non alcoholic liver steatosis (NASH), cholestasis.

INTRODUCTION

The farnesoid X receptor (FXR), a member of the nuclear receptor superfamily of ligand-activated transcription factors, is a bile sensor [1]. It forms part of a complex network of nuclear receptors that act co-ordinately to regulate essential steps of bile acid and xenobiotic uptake, metabolism and excretion by hepatocytes, cholangiocytes and kidney cells [2-4]. The FXR is primarily expressed in the liver, intestine, kidney and adrenal glands with much lower levels in adipose tissue [1-4]. Members of the nuclear receptor superfamily share a common modular structure comprising a highly conserved DNA-binding domain in the N-terminal region and a moderately conserved ligand-binding domain in the C-terminal region [5]. The FXR is an obligate partner of the 9-*cis*-retinoic acid receptor (RXR) and FXR/RXR heterodimers bind DNA sequences that are typically composed of two inverted repeats (IR) separated by one nucleotide, IR-1. Upon ligand binding, the FXR undergoes conformational changes to release corepressors and to recruit coactivators, the best known of which is the steroid receptor coactivator-1 [5]. The mechanism(s) that modulate recruitment of these coactivators by FXR ligands and the relevance of these molecules to the regulation of specific genes by FXR ligands in specific tissues are largely unknown [5, 6]. Two primary bile acids, chenodeoxycholic acid (CDCA) 1 and cholic acid (CA) 2, are the most potent endogenous ligands for the FXR with an EC_{50} value for CDCA 1 of ~ 10 μM [5, 6]. Secondary bile acids such as deoxycholic acid (DCA) 3 and lithocholic acid (LCA) 4 are weak FXR agonists or partial antagonists [6]. Synthetic FXR agonists, 6 ethyl-CDCA 5, GW4064 6, fexaramine 7, AGN-31 8 and AGN 34 9 are shown in Fig. (**1**). Although there is a growing body of evidence linking FXR activity to modulation of key aspects of lipid and glucose metabolism [7-9], the FXR was originally characterized for its ability to influence the expression of genes involved in bile acid synthesis and detoxification [1]. Transcriptional activation of the FXR is triggered by a rise in the intracellular concentration of bile acids (Fig. **2**). One hepatic FXR target gene is the small heterodimer partner (Shp), which dimerizes with and inactivates two positive regulators of bile acid transport, liver receptor homolog 1 and liver X receptor α, leading to diminished expression of cholesterol 7 α-hydroxylase (Cyp7a1) and inhibition of bile acid synthesis through the neutral pathway [10]. Conversely, FXR activation negatively regulates basolateral hepatocyte bile acid uptake *via* repression of sodium-dependent taurocholate cotransporter (Ntcp) and organic anion transporter protein (Oatp)1 and 4, and also by stimulating overall gene expression of both canalicular (multidrug resistance-associated protein [MRP]2 and the bile salt export pump [BSEP] and alternative basolateral efflux transporters

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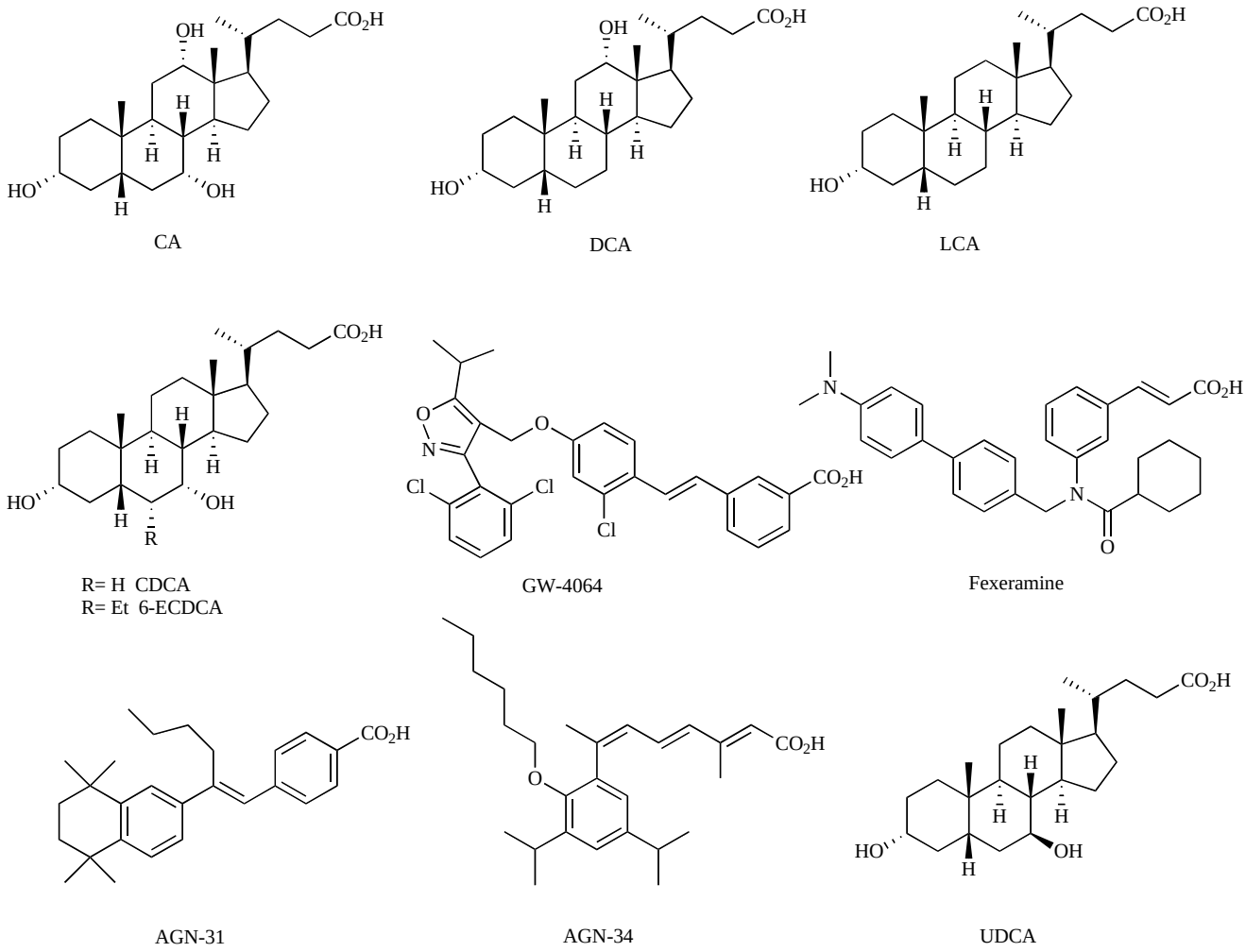


Fig. (1). Natural and synthetic FXR ligands.

(multidrug resistance protein 3 [MDR3] P-glycoprotein, MRP3 and organic solute transporter (Ost)α and β) [11]. In addition to SHP, FXR influences the expression of other nuclear receptors including the pregnane X receptor (PXR) and PPARα and γ [7,12-14]. These interactions might account for the pleiotropic activity of the FXR in tissues where the expression of this receptors is low [7,12-14].

CHOLESTATIS: CLINICAL RELEVANCE AND TREATMENT OPTIONS

Several liver disorders are characterized by intrahepatic cholestasis (ie, the impaired secretion of bile constituents); in primary billiary cirrhosis (PBC), cholestasis is the main clinical feature, while in other settings, such as HBV and HCV induced chronic hepatitis or cirrhoses of any etiology, cholestasis develops as a consequence of progressive disarray of liver architecture [3]. The most common cause of cholestasis is PBC, a disease that is characterized by the slow but progressive destruction of the small bile ducts within the liver [3, 15, 16]. Current therapeutic options for PBC are limited [17]. Ursodeoxycholic acid (UDCA) **10** is the principle medication used to slow the progression of PBC [18]. There is some evidence that UDCA **10** treatment prolongs transplant-free survival [17-21] and might slow down the progression of histology lesions [22,23]. However, meta-analyses have been inconsistent and some have concluded that UDCA **10** has no beneficial effect on the incidence of death and/or transplant survival despite improvements in serum bilirubin levels, jaundice and ascites [24,25]. Despite additional therapies are available for treating pruritus (antihistamines, cholestyramine and colestipol and rifampicin) their use is frequently associated with side effects [14,26] or poor clinical outcomes [14].

6-ethyl-CDCA as FXR Agonist

6E-CDCA **5** (also called INT-747) (Figs. **3** and **4**), being developed by Intercept Pharmaceuticals Inc, is an FXR agonist for the oral treatment of cholestatic liver diseases including PBC, non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH) [27,28]. The FDA and the EMEA have granted this agent the orphan drug status for the treatment of PBC [29-31]. So far the 6E-CDCA **5** had been evaluated in two phase I clinical trials in healthy volunteers and two phase II trials in patients with type 2 diabetes mellitus and NAFLD (ClinicalTrials.gov identifier: NCT00501592) and PBC (NCT00550862), respectively [32-35]; in addition, a phase II trial (NCT00570765) in patients

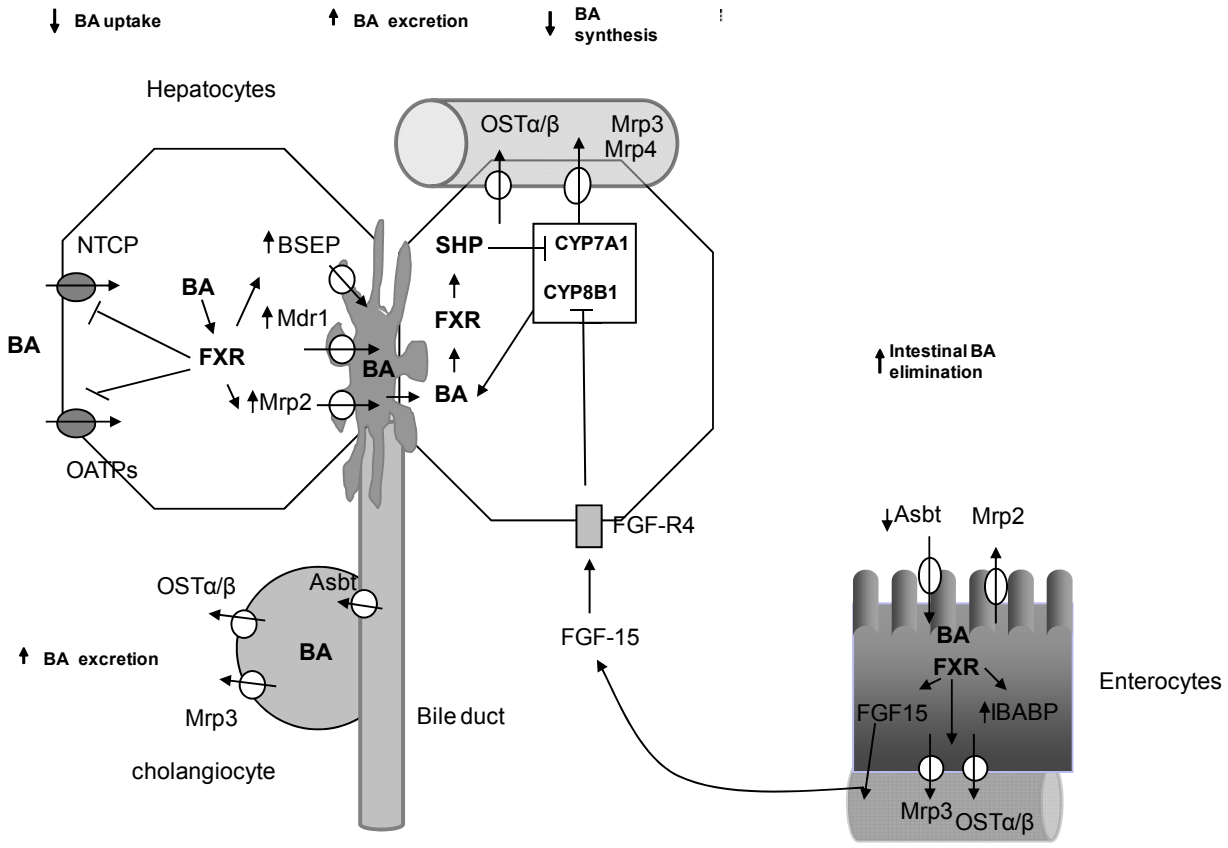


Fig. (2). Biochemical effects of FXR activation.

Abbreviations used are: Asbt= apical sodium-dependent bile acid transporter; BA= bile acid; BSEP (ABCB11/Abcb11)=bile-salt export pump; CYP7A1=cholesterol 7α-hydroxylase; CYP8B1=sterol 12β-hydroxylase; FGF15= fibroblast growth factor 15; FGFR4= fibroblast growth factor receptor 4; FXR (NR1H4)=farnesoid X receptor; IBABP= ileal bile acid-binding protein; Mdr1, Mdr2 (ABCB/abcb)= Multi-Drug Resistance 1,2; Mrp1 Mrp2 Mrp3 and Mrp4(ABCC/abcc)= Multidrug Resistance Associated Protein 1,2,3,4; NTCP(SLC10A1/Slc10a1)=Na+ taurocholate cotransport peptide; Oatps (SLCs)= organic anion transporting proteins; Ostα, Ostβ= organic solute transporter α and β; SHP (NR0B2)= short heterodimer partner.

with PBC is ongoing, and additional trials have been planned [35-37].

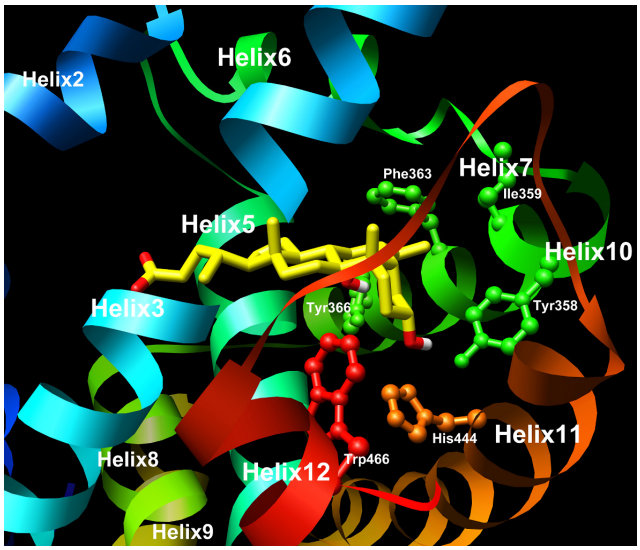
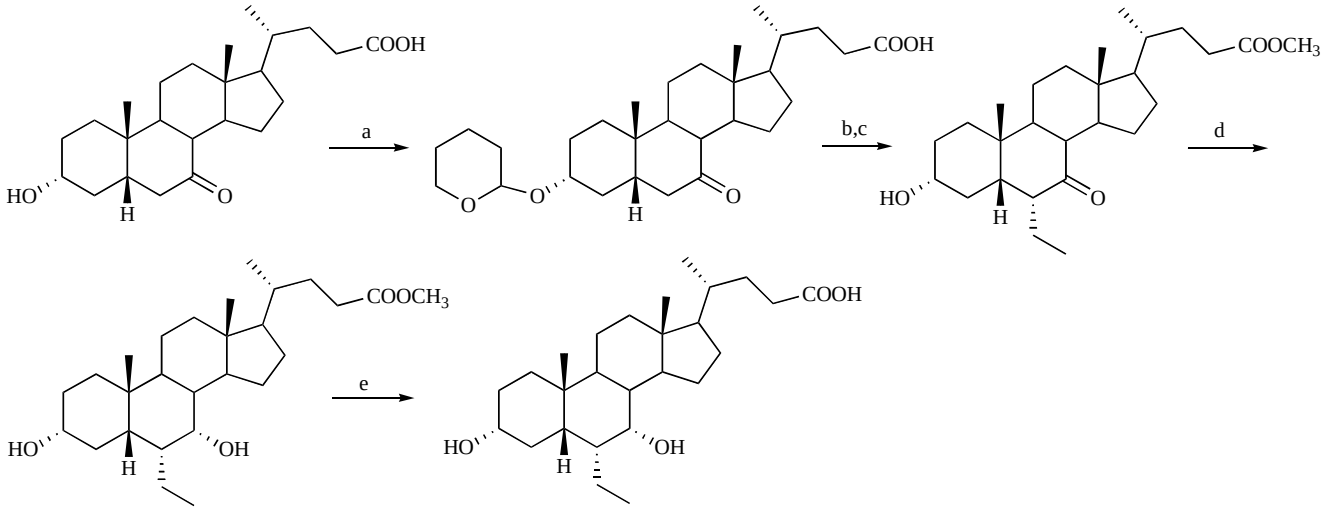


Fig. (3). Structure of FXR and its ligand binding domain: 6E-CDCA is shown in yellow.

6E-CDCA **5** was originally synthesized from 7-keto-LCA (Fig. **4**), which could be obtained *via* selective oxidation of the 7-hydroxy group of CDCA **1** [38], [39]. The 3-tetrahydropyranyloxy derivative of 7-keto-LCA (prepared using standard procedure) was treated successively with lithium diisopropylamide and ethyl bromide in THF at -78°C, and then refluxed with methanolic HCl (10%) to yield methyl 3*R*-hydroxy-7-keto-6*R*-ethyl-5β-cholan-24-oate. The 7-keto group was reduced with sodium borohydride and the 24-methyl ester was hydrolyzed with 10% sodium hydroxide in methanol to produce 6E-CDCA **5** in good yield [38,39]. A similar, economical and large-scale preparation was published in US-20090062526, while an alternative procedure *via* 3-alpha-hydroxy-6-ethylidene-7-keto-5β-cholanic acid was provided in WO-2006122977. Combination therapies, new uses and product derivatives were described in WO-2006044391, WO-2005089316 and WO-2005082925, respectively.

6E-CDCA 5 is and FXR ligand designed to obtain a better occupancy of the FXR ligand binding domain [40]. The structure of the FXR ligand binding domain (Fig. 3) is referred to as an α-helical sandwich in which three antiparallel α-helices (the 'sandwich filling') are flanked by two α-



a) pTsOH, 3,4-dihydropyrane, dioxane, 25 °C; b) LDA, EtBr, THF, -78 °C; c) 10% HCl, MeOH, reflux; d) $NaBH_4$, MeOH, 0°C; e) 10% NaOH, MeOH, r.t..

Fig. (4). Synthetic strategy for 6-ECDCA.

helices on one side and three on the other (the 'bread') [40]. The ligand binding cavity is located within the interior of the ligand binding domain and just below the α-helical sandwich 'filling' [41]. Along with the DNA binding domain, the ligand binding domain contributes to the dimerization interface of the FXR, and in addition, binds coactivator and corepressor proteins [42-43]. The disclosure of crystallographic structures of the FXR ligand binding domain complexed with either 6E-CDCA 5 or the nonsteroid ligand fexaramine 7 has helped to answer some structural and mechanistic questions [28-30, 41]. 6E-CDCA 5 is a water-soluble amphipathic steroid and the physicochemical properties of its bile acid skeleton are essential for recognition by the FXR. The steroid nucleus in 6E-CDCA 5 is not flat because a hydrogen atom oriented in the β-configuration at C5 causes the A/B ring juncture to be *cis*, forcing ring A to lie externally of the plane of the BCD ring system [28-30]. As a result, the separation between the 3α-hydroxyl and the C24 carboxylate of CDCA 1 is shorter than the contour length of the molecule, which endows it with a rounded profile allowing a close fit with respect to the FXR binding pocket (Fig. **3**). The FXR ligand binding cavity also employs the amphipathic properties of bile acids to provide additional molecular recognition beyond their unique shape [28-30, 41]. Indeed, the FXR structure reveals pockets that are not entirely filled by naturally occurring bile acids [40-43]. In the case of 6E-CDCA 5, the 6α-ethyl group is located within one such hydrophobic cavity that exists between the side chains of Ile^{359}, Phe^{363} and Tyr^{366}, accounting for its higher affinity (Fig. **3**). 6E-CDCA 5 interacts with the FXR ligand binding domain with ring A directed toward helix 11 and 12 of the binding domain, while the carboxylic acid function of the side chain approaches the entry pocket at the back [28-30, 40,41]. This disposition is different from that adopted by other cholesterol metabolites that bind to their cognate receptors with the oxidized tail towards helix 12. Furthermore, ring A is not directly in contact with helix 12, but instead stabilizes its 'active' disposition through a triad of residues Trp^{466} (helix 12), His^{444} (helix 11) and Tyr^{358} (helix 10). UDCA 10 (Fig. **1**) has two hydroxyl groups oriented in *trans* rather than in *cis*, such that UDCA 10 creates a more open ligand binding pocket, and this arrangement may force a suboptimal orientation of helix 12 and results in partial inhibition [28-30, 40,41].

PRECLINICAL DEVELOPMENT

Animal Models of Cholestasis

In vitro, 6E-CDCA **5** activates the FXR with an EC_{50} value ranging from 100 to 300 nM [44,45]. A 24-h incubation of rat hepatocytes with 6E-CDCA **5** (1 μM) resulted in 3- to 5-fold higher mRNA expression of SHP and BSEP, and 50 to 70% reduction in the expression of Cyp7a1, oxysterol 12α-hydroxylase (Cyp8b1) and NTCT ($p < 0.05$ compared with control cells) [46]. *Oatp1* gene expression was not altered by exposure to 6E-CDCA [46]. The potential *in vivo* anti-cholestatic effects of 6E-CDCA **5** (Fig. **2**) were initially investigated in intact rats and in rats with an acute biliary external fistula [47]. In intact rats, 6E-CDCA **5** (1 or 5 mg/kg po, daily for 5 days) did not increase serum levels of γ-glutamyl transpeptidase, ALP, cholesterol or bilirubin. In rats with a biliary fistula, co-infusion of 6E-CDCA **5** (3 μM/kg/min) with LCA **4** (3 μM/kg/min), prevented LCA-induced cholestasis [47]. Another experiment using intact rats demonstrated that 6E-CDCA (1 or 5 mg/kg for 7 days) reduced fecal excretion of taurocholic acid and tauro-β-muricholate by 50 to 60% [45].

Further experiments were conducted in a rat model of estrogen-induced cholestasis [46], [45]; however, it should be noted that unlike the cholestasis observed in PBC, changes in liver morphology in estrogen-induced cholestasis are absent, therefore this model is not predictive of results in PBC [46]. Administering male rat with 17α estradiol (5 mg/kg ip, daily for 5 days) causes a light increase in f serum ALP ($p < 0.05$ compared with untreated control rats) and bile acids, but no changes in γ-glutamyl transpeptidase, ALT or

bilirubin levels [46]. Coadministration of 6E-CDCA **5** (10 mg/kg ip, daily for 5 days) reduced serum ALP activity ($p < 0.05$ compared with estrogen alone) and dose-dependently improved the cholestatic changes caused by estrogen ($p < 0.01$ compared with estrogen alone). 6E-CDCA **5** partially abrogated the estrogen-induced reduction in total bile acid output as indicated by the increased relative abundance of taurochenodeoxycholic acid and taurodeoxycholic acid (both $p < 0.05$ compared with estrogen alone). 6E-CDCA **5** increased mRNA expression of SHP and decreased NTCP, CYP7A1 and CYP8B1 ($p < 0.01$ compared with estrogen alone). Further on, administration of 6E-CDCA **5** was associated with 2- to 4-fold increase in the expression of BSEP, MRP2 and MDR2 ($p < 0.05$ compared with control rats). These changes were not reproduced by CDCA **1** [46].

We have also shown that in a human hepatocyte cell line (HepG2), exposure to 6E-CDCA **1** correlated with 30-40 folds increase in the expression of *OSTα* and *OSTβ*, [44]. In wild-type, but not *Fxr*$^{-/-}$ mice, treatment with 6E-CDCA **5** (5 and 10 mg/kg ip, daily for 5 days) induced Ostα and Ostβ mRNA expression. A subsequent transactivation assay demonstrated that 6E-CDCA **5** activates the *OSTα* and *OSTβ* promoters. In a mouse model of estrogen-induced cholestasis, expression of Ostα and Ostβ was down-regulated and alleviation of cholestasis with 6E-CDCA **5** (5 and 10 mg/kg ip, daily for 5 days) correlated with increased expression of both transporters [44].

Animal Models of NASH/NALFD

We have shown that exposure of preadipocytes (3T3-L1 cell line; cultured in a differentiating medium) to 6E-CDCA **5** (1 μM for 8 days) resulted in enhanced cell differentiation and correlated with the induction of aP2, CCAAT/enhancer-binding protein α and PPARγ2 mRNA expression, as well as that of other adipocyte-related genes [48]. Furthermore, insulin-induced Akt phosphorylation and glucose uptake was increased in preadipocytes incubated with 6E-CDCA **5** [48]. Treatment of mouse pancreatic β-TC6 cells or human pancreatic islets with 6E-CDCA **5** enhanced insulin secretion by β-TC6 cells correlated with an increase in Akt phosphorylation and translocation of glucose transporter 2 from the plasma membrane. In accordance with the above data, 6E-CDCA **5** improved glycemia in a glucose tolerance test in wild-type mice ($p = 0.02$ compared with *FXR*$^{-/-}$ mice) and also in wild-type, but not *Fxr*$^{-/-}$ mice, 6E-CDCA stimulated a minor increase in plasma insulin levels [49]. Zucker rats develop diabetes, insulin resistance, obesity and liver steatosis, symptoms that are associated with NAFLD and NASH [6]. We have investigated whether administering Zucker rats with 6E-CDCA **5** (10 mg/kg po, qd for 7 weeks), rosiglitazone (10 mg/kg po, qd for 7 weeks), or 6E-CDCA **1** (5 mg/kg po, qd for 7 weeks) in combination with rosigliatzone (5 mg/kg po, qd for 7 weeks) attenuates liver steatosis. After 7 weeks of treatment, the body mass of rats treated with 6E-CDCA **5** alone was unaltered from baseline, whereas rats treated with rosiglitazone, or 6E-CDCA **5** and rosiglitazone, displayed an increase of ~ 20%, compared with an increase of ~ 10% in the body mass of untreated Zucker rats. In addition, compared with untreated rats, treatment with 6E-CDCA **5** alone was associated with a decrease in plasma glucose ($p < 0.05$), free fatty acid and HDL ($p < 0.05$) levels, as well as in the triglyceride, free fatty acid, cholesterol and glycogen content of the liver ($p < 0.05$). Histological analysis of liver samples also demonstrated that 6E-CDCA **5** reduced triglyceride levels and improved the obesity-induced pathology, whereas rosiglitazone had the opposite effect. 6E-CDCA **5** elicited an increase in SHP expression and reduced the expression of genes involved in fatty acid synthesis (sterol regulatory element-binding protein-1c, Fas, malic enzyme and pyruvate kinase) and neoglucogenesis (phosphoenolpyruvate carboxykinase); in addition, apolipoprotein C2 was upregulated. In muscle tissue, 6E-CDCA **5** treatment reduced triglyceride, free fatty acid and cholesterol levels, by downregulating Fas mRNA while glucose transporter (GLUT)-4, PPARα and PPARγ coactivator-1α were induced. In 22-week-old Zucker rats, administration of 6E-CDCA **5** with rosiglitazone, or either agent alone, normalized plasma glucose and insulin levels and improved insulin sensitivity in an insulin tolerance test. In addition, Zucker rats display aberrant insulin signaling and this was also improved by treatment with 6E-CDCA **5**, rosiglitazone or both agents together, as illustrated by reduced IRS phosphorylation in Ser^{312} and increased Akt phosphorylation in Ser^{437} [7]. In non-obese diabetic mice, a model of type 2 diabetes mellitus, administration of 6E-CDCA **5** for 12 weeks protected against diabetes development [49]. Similarly to these data, administering 6E-CDCA **5** to male DBA/2J mice fed a Western high-fat, high-cholesterol diet an yielded a reduction in plasma cholesterol, HDL-cholesterol and LDL-cholesterol as well as ameliorating the increase in the LDL-cholesterol/total cholesterol ratio that was observed in untreated Western diet-fed animals These changes were not observed in *FXR* $^{-/-}$ mice [50].

We have also shown that In *Apoe*$^{-/-}$ mice, 6E-CDCA **5** (3 and 10 mg/kg, daily for 12 weeks) dose-dependently attenuated aortic plaque formation with the 10-mg/kg dose yielding a reduction of ~ 95% in plaque size ($p < 0.01$ compared with untreated mice) [51]. Administration of 6E-CDCA **5** (10 or 20 mg/kg for 12 weeks) to *Apoe*$^{-/-}$ mice with chronic kidney disease (induced by nephrectomy) resulted in a reduction in both serum and hepatic triglycerides, and hepatic cholesterol levels were reduced with the 20- but not the 10-mg/kg dose [52]. Atherosclerotic plaque formation was not affected by 6E-CDCA **5** but a 61 to 66% reduction in aortic calcium content was observed in treated as compared with untreated *Apoe*$^{-/-}$ mice with chronic kidney disease. Further, the calcified lesion area was 81% lower in mice treated with the 10-mg/kg dose, compared with untreated mice [52].

Animal Models of Liver Fibrosis

FXR activation has previously been demonstrated to increase PPARγ mRNA expression in human hepatocytes and, in addition, activation of PPARγ is associated with protection against liver fibrosis *in vivo* [53]. In primary rat hepatic stellate cells, exposure to 6E-CDCA **5** (1 μM for 7 days) resulted in 40-fold higher mRNA and protein expression of PPARγ ($p < 0.01$ compared with vehicle control). Further assays in a rat hepatic stellate cell line (HSC-T6) demonstrated that a 24-h incubation with either 6E-CDCA **5** (1 μM) or the Pparγ agonist rosiglitazone (10 μM) yielded a 90% reduction in TGFβ1-induced upregulation of α1(I) collagen mRNA expression; IC_{50} values against TGFβ1-induced col-

lagen upregulation were 0.08 and 3.3 μM for 6E-CDCA **5** and rosiglitazone, respectively. Exposure of HSC-T6 cells with 6E-CDCA **5** (0.1 μM) and rosiglitazone (1 μM) produced 3-fold higher PPARγ mRNA expression and ~ 80% lower α1(I) collagen and α-Sma mRNA expression ($p < 0.05$ compared with either agent alone) [53]. Also, in primary rat hepatic stellate cells, 6E-CDCA **5** reduced thrombin-induced upregulation of α1(I) collagen, α-Sma, Timp-1, Timp-2 and matrix metalloproteinase (MMP)-2 mRNA expression and attenuated thrombin-induced Timp-1 secretion by 50 to 80% ($p < 0.01$ compared with thrombin) [54]. In a human hepatic stellate cell line (LX2), incubation with 6E-CDCA **5** (3 mM for 1 h) reduced mRNA expression of α-SMA, TGFβ1, Mmp-2, Timp-1 and Timp-2 [55].

The potential of 6E-CDCA **5** to ameliorate liver disease has also been investigated in three different rat models of liver fibrosis: porcine serum administration, bile duct ligation (BDL) and carbon tetrachloride (CCl_4) intoxification [54-56]. For each type of model, hepatic stellate cell cultures obtained from control fibrotic rats were compared with those derived from fibrotic rats that had been treated with 6E-CDCA **5** (3 mg/kg po, 5 times a week for 8 weeks; 3 mg/kg po for 14 days; 3 mg/kg po, 5 times a week for 8 weeks, for each respective model) [53]. Cultures of hepatic stellate cells derived from control fibrotic rats demonstrated that PPARγ expression was almost undetectable ($p < 0.05$ compared with non-fibrotic rats). In contrast, hepatic stellate cell cultures derived from fibrotic rats treated with 6E-CDCA **5** exhibited 30- to 50-fold higher PPARγ expression ($p < 0.01$ compared with control fibrotic rats), and in addition, FXR and Shp mRNA levels were 1.8- to 4-fold greater compared with cultures derived from control fibrotic rats. Compared with administration of porcine serum alone, 6E-CDCA yielded a 50 to 60% reduction in the area of fibrotic parenchymal liver tissue, hepatic hydroxyproline levels, and mRNA expression of α-Sma, α1(I) collagen, fibronectin, TGFβ1 and tissue inhibitors of matrix metalloproteinases (Timp)-1 and Timp-2 ($p < 0.01$ for all parameters). Coadministration of 6E-CDCA and rosiglitazone (3 mg/kg) enhanced the antifibrotic effect compared with that of either drug alone [53].

Interestingly it appears that 6E-CDCA **5** (3 mg/kg po) might accelerate collagen removed in CCl_4 intoxicated rats [54]. The antifibrotic effects of 6E-CDCA **5** have also been investigated in rats that were receiving concurrent treatment with thioacetamide (group 1), or in rats with established thioacetamide-induced fibrosis (group 2) or cirrhosis (group 3) [55]. 6E-CDCA **5** also resulted in lower portal pressure in each of the three groups of rats ($p < 0.05$ for groups 1 and 2 and $p < 0.01$ for group 3, all compared with control thioacetamide-treated rats). No change in serum ALT or AST levels was observed following 6E-CDCA treatment [55].

Data from patients with cirrhosis suggested that an agonist of dimethylarginine dimethylaminohydrolase (DDAH)-1 may reduce portal pressure by restoring defective endothelial nitric oxide synthase (NOS) activity [57]. Therefore, because DDAH-1 is one of the target genes of FXR activation, the effects of 6E-CDCA (5 mg/kg po, daily for 5 days) on portal pressure were assessed in bile duct ligated rats. Histological assessment indicated that the degree of liver injury and fibrosis was similar in rats treated with either vehicle or 6E-CDCA **5**. Portal pressure was lower in rats treated with 6E-CDCA **5** ($p = 0.002$ compared with vehicle), and not different ($p = 0.2$) from rats that had undergone sham bile duct ligation [57]. In addition, administration of 6E-CDCA **5** (5 mg/kg po, daily for 3 days) to rats treated with CCl_4 protects against CCl_4-induced downregulation of cystathionase, a gene required for hydrogen sulphide (H_2S) production. Because, H_2S is an essential mediator of the hepatic microcirculation the reduced production of H_2S in this model might increase intrahepatic resistance. We have found that FXR activation increases CSE expression and H_2S generation [58].

Animal Models of Intestinal Inflammation and Fibrosis

The anti-inflammatory effects of 6E-CDCA has been demonstrated in rodent models of colitis [59-[61]. In these models, 6E-CDCA **5** (5 mg/kg po, daily for 8 weeks) pretected against development of trinitrobenzenesulfonic acid-induced colitis in BALB/c mice, whereas virtually all the control animals administered trinitrobenzenesulfonic acid alone, displayed mild-to-moderate fibrosis [61].

Animal Toxicity, Metabolism and Pharmacokinetics

Preclinical toxicity data for 6E-CDCA **5** are not available. In animals we have found that that 6E-CDCA is almost completely absorbed by the intestine, taken up by the liver and secreted into the bile after conjugation with taurine [46]. 6E-CDCA **1** is secreted into the bile as tauro-6-ethyl-CDCA and reabsorbed in the distal ileum from where it undergoes enterohepatic circulation as the parent compound, CDCA **1**, does [46]. Administering rats with 6E-CDCA **5** dramatically alters the bile acid pool with a marked reduction in the concentration of CA **2** and CDCA **1**, and a notable increase in the concentration of taurine-6-ethyl-CDCA (~ 70% of total bile acids detected in the bile after 5 days). Thus, in rats, all of the pharmacological activity of 6E-CDCA **5** is mediated by its taurine conjugate [46].

The pharmacokinetic profile of 6E-CDCA in humans is likely to be similar to that observed in animals. However, because bile acids in humans are preferentially conjugated to glycine, the main circulating metabolite will be glycine-6-ethyl-CDCA **1** [46]. In terms of pharmacological activity, it is likely that conjugation will not affect the ability to bind and activate the FXR. The concentration of 6E-CDCA **5** that is required to produce activation of the FXR *in vivo* is unknown.

Metabolism in Humans

Two phase I, double-blind, placebo-controlled, dose-escalation clinical trials evaluated the pharmacokinetics of single-ascending and multiple-ascending doses of 6E-CDCA **5** (50, 100, 250 or 500 mg po, and 25, 50, 100 or 250 mg po, qd for 12 days, respectively) in healthy volunteers ($n = 74$) [33]. Following single or multiple doses of 6E-CDCA **5**, maximum and total drug exposure was dose-proportional. 6E-CDCA was absorbed rapidly, metabolized to glycine and taurine conjugates and excreted *via* the liver. Plasma concentration-time profiles revealed multiple peaks, which suggested reabsorption of 6E-CDCA **5** because of prolonged enterohepatic recirculation [33].

Clinical Trials in PBC Patients

A phase II, randomized, double-blind, placebo-controlled, parallel-group-assignment, dose-response, international clinical trial (NCT00550862) evaluated 6E-CDCA **5** (10, 25 or 50 mg po, qd for 12 weeks) in patients (n = 165) with PBC who continued to receive a stable dose UDCA **10** [35]. A decrease in ALP levels of 24, 25 and 21% was observed in the 10-, 25- and 50-mg groups ($p < 0.0001$ for all dose groups compared with placebo). Levels of gamma glutamyl transpeptidase were also reduced, by 48, 63 and 57% in each respective dose group ($p < 0.0001$ for all dose groups compared with placebo), and levels of ALT were reduced by 28, 35 and 21% in each respective dose group ($p < 0.0001$ for the 10 and 25-mg groups and$p < 0.0005$ for the 50-mg group, all compared with placebo) [35].

A phase II, randomized, double-blind, placebo-controlled, parallel-group-assignment clinical trial (NCT00570765) of 6E-CDCA **5** (10 or 50 mg po, qd for 12 weeks) was recruiting patients (expected n = 120) with PBC. The primary endpoint was to monitor ALP levels and safety. Secondary endpoints were to assess hepatocellular injury and liver function, disease-specific and general health symptoms, biomarkers of hepatic inflammation and fibrosis, and to determine trough plasma concentrations of 6E-CDCA **5** and its known metabolites. At the time of publication, trial completion was scheduled for December 2010.

Clinical Trials in Diabetic Patients

Phase II, randomized, double-blind, placebo-controlled, parallel-group-assignment, multiple-dose, exploratory clinical trial (NCT00501592) evaluated the effects of 6E-CDCA **5** (25 or 50 mg po, qd for 6 weeks) on insulin sensitivity, in patients (n = 64) with type 2 diabetes mellitus and NAFLD [32], [34]. The glucose disposal rate was measured pre- and post-treatment after steady-state was attained following low- and high-dose (60 and 120 mU × body surface area [m^2]/min, respectively) insulin infusions [34]. The 25-mg dose of 6E-CDCA **5** increased the glucose disposal rate after low- and high-dose insulin (both $p < 0.05$ compared with placebo), and also resulted in higher fasting plasma insulin concentrations ($p = 0.085$ compared with placebo). Body mass was reduced by 1.7% after treatment with the 50-mg dose of 6E-CDCA **5** ($p < 0.01$ compared with placebo). In the enhanced liver fibrosis test, the 25-mg dose of 6E-CDCA **5** was associated with improvements in each of the three test components, as well as in the total score ($p < 0.05$ compared with placebo). FGF-19 levels were enhanced by ~ 60 and 120% in the 25- and 50-mg 6E-CDCA **5** groups, respectively ($p = 0.05$ and 0.01, respectively, compared with placebo). In addition, both doses of 6E-CDCA **5** yielded a 50% reduction in levels of gamma glutamyl transpeptidase (an indicator of biliary tract health; $p < 0.001$ compared with placebo), while the 25-mg dose resulted in a reduction of ~ 25% in ALT levels ($p < 0.001$ compared with placebo) [34].

Clinical Trials. Side Effects

In the phase I single- and multiple-ascending-dose clinical trials in healthy volunteers, no serious adverse events were reported. Single doses of up to 500 mg and multiple doses of up to 100 mg of 6E-CDCA **5** were well tolerated [33]. Elevations in both ALT and AST levels were detected in 4/16 and 4/8 individuals in the 100- and 250-mg multiple-dose group, respectively, and in addition, elevated ALT levels only were detected in 2/8 individuals in the 250-mg multiple-dose group. The highest liver aminotransferase level (209 IU/l) was ~ 5 × the upper limit of normal but levels decreased rapidly after discontinuation of 6E-CDCA **5**. The most frequent adverse event was pruritis, which was reported by 4/8 individuals in the 250-mg multiple-dose group. With the exception of headache (n = 4, of whom 3 were in the placebo group), the only other adverse events reported by more than one individual were pharyngolaryngeal pain (n = 2), nausea (n = 2) and upper abdominal pain (n = 2), which were all observed in individuals in the 100- and 250-mg multiple-dose groups. One individual in the 250-mg multiple-dose group discontinued the trial because of a skin rash on day 7 [33].

In the NCT00501592 clinical trial in patients with type 2 diabetes mellitus and NAFLD, the majority of adverse events were of mild-to-moderate severity and the frequency of adverse events was similar in the placebo (61%) and the 25- and 50-mg 6E-CDCA **5** groups (45 and 76%, respectively) [32], [34]. The most common adverse event was constipation (24% of patients in the 50-mg dose group) [34]. In addition, a preliminary report of the data from this trial stated that minor increases in LDL levels were observed in the 25- and 50-mg groups, and minor decreases in HDL and triglyceride levels were observed in the 50-mg group [32].

In the NCT00550862 clinical trial in patients with PBC, the majority of adverse events were of mild-to-moderate severity with the most frequent being pruritus [35]. In the placebo, 10-, 25- and 50-mg group, pruritis was experienced by 50, 47, 85 and 80% of patients, respectively, and the trial was discontinued because of pruritis by 0, 8, 8 and 24% of patients in the respective groups. Seven patients (n = 1 each in the placebo and 25-mg group and n = 5 in the 50-mg group) reported a serious adverse event, of which three (all in the 50-mg group and all resolved) were hepatic (gastrointestinal bleed from prior varices, PBC flare and jaundice) [35].

FINAL CONSIDERATIONS

PBC is a condition for which available therapies do not provide adequate control over disease progression and liver transplant remains the only life-saving procedure. 6E-CDCA **5** has demonstrated some utility for the treatment of cholestasis in the estrogen model in rodent. However, data derived from more robust rodent models of cholestasis raise concern over the use of an FXR agonist in obstructive cholestasis. Indeed, it is increasingly recognized that activation of bile acid secretion and impairment of basolateral transporters might exacerbate liver injury in a condition of bile flow obstruction. PBC is characterized by progressive bile duct destruction and ineffective bile duct proliferation, causing bile duct loss and progressive bile flow impairment. Patients with late-stage PBC display severe impairment of bile flow and activation of the FXR in could lead to side effects. Therefore, in the author's opinion, the use of an FXR agonist needs to be reserved for carefully selected patients, probably those with early-stage disease. However, the increased inci-

dence of pruritus in the phase II clinical trial in patients with PBC raises a red flag with regard to the clinical potential of this agent, even in patients with early-stage PBC. The fact that nearly all patients reported a clinically significant exacerbation of pruritus during treatment with 6E-CDCA **5** (25 or 50 mg/kg po, qd) makes the development this agent for PBC challenging. Furthermore, the incidence of pruritus increased dose-dependently, indicating that this side effect is linked mechanistically to tissue accumulation of 6E-CDCA **5,** FXR activation, or both.

In animal studies we have recently shown that FXR functions as a CAR antagonist in regulating the expression of the basolateral transporter MRP4 [62]. Impairment of this transporter might be a drawback in the use of FXR agonists in PBC. An additional consideration should be made taking into account that in the above mentioned studies 6E-CDCA **5** was used in combination with UDCA **10**. UDCA **10** shifts the bile pool to more hydrophilic bile acids, leading to a reduction in levels of CDCA **1** and CA **2**, which are both hydrophobic molecules. 6E-CDCA **5** is a hydrophobic bile acid, and therefore, the effect of this agent when administered alone (ie, in the absence of UDCA **10**) needs to be assessed because of the potential to shift the bile acid pool towards hydrophobic bile acids.

Despite the fact that 6E-CDCA **5** met the primary endpoint of a reduction in ALP levels in the phase II clinical trial, it is unknown whether it has any impact on clinically meaningful endpoints such as liver histology, increased survival or delay in transplant time. Indeed, the advantage of reducing levels of a surrogate marker such as ALP should be viewed in the context that 6E-CDCA **5** exacerbated the main symptoms of PBC, resulting in up to 25% of patients discontinuing treatment. This lack of patient compliance should be considered in the context of the exploitation of the orphan indication for PBC. Indeed, the grant of an accelerated track based on a surrogate marker appears to be justified only if the hypothetical benefits are not associated with safety and tolerability issues. Despite the results in patients with PBC, 6E-CDCA **5** may be efficacious in the treatment of other conditions characterized by bile flow impairment, such as pregnancy-induced cholestasis and drug-induced cholestasis. Clinical trials are needed to prove the efficacy of 6E-CDCA in these settings. Administration of 6E-CDCA **5** improved insulin sensitivity in patients with type 2 diabetes mellitus and suspected NAFLD. This effect is clinically promising and is mechanistically supported by preclinical studies that have demonstrated a role of the FXR in regulating insulin secretion and sensitivity [49]. Currently, the use of 6E-CDCA in the treatment of NAFLD/NASH holds promises that have been partially fulfilled by the results of the phase II clinical trial (NCT00501592). The list of drugs that have been displayed some efficacy in the treatment of NAFLD/NASH is growing; for example, a recent meta-analysis listed 49 randomized trials designed to assess different treatment modalities in patients with NAFLD [63]. The use of insulin sensitizers in the treatment of NASH remains controversial. In a recently published trial (NCT00063622), administration of the PPARγ agonist pioglitazone failed to meet the primary outcome, an improvement in histological features of NASH, as assessed with the use of a composite of standardized scores for steatosis, lobular inflammation, hepatocellular ballooning and fibrosis [63].

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