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Novel FXR (Farnesoid X receptor) Modulators: Potential Therapies for Cholesterol Gallstone Disease

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ABSTRACT:

Metabolic disorders such as diabetes are known risk factors for developing cholesterol gallstone disease (CGD). Cholesterol gallstone disease is one of the most prevalent digestive diseases, leading to considerable financial and social burden worldwide. Ursodeoxycholic acid (UDCA) is the only bile acid drug approved by FDA for the non-surgical treatment of gallstones. However, the molecular link between UDCA and CGD is unclear. Previous data suggest that the farnesoid X receptor (FXR), a bile acid nuclear receptor, may protect against the development of CGD. In studies aimed at identifying the role of FXR, we recently identify a novel chemical tool, 6EUDCA (6-α ethyl-ursodeoxycholic acid), a synthetic derivative of UDCA, for studying FXR. We found that 6EUDCA binds FXR stronger than UDCA in a TR-FRET binding assay. This result was supported by computational docking models that suggest 6EUDCA forms a more extensive hydrogen bound network with FXR. Interestingly, neither compound could activate FXR target genes in human nor mouse liver cells, suggesting UDCA and 6EUDCA activate non-genomic signals in an FXR-dependent manner. Overall these

studies may lead to the identification of a novel mechanism by which bile acids regulate cell function, and 6EUDCA may be an effective targeted CGD therapeutic.

ABBREVIATIONS USED

FXR, Farnesoid X Receptor; CDCA, chenodeoxycholic acid; UDCA, Ursodeoxycholic acid; 6EUDCA, 6-αethyl-ursodeoxycholic acid; qPCR, quantitative polymerase chain reaction; TGR5, G-protein-coupled receptor.

1. Introduction

Metabolic disorders such as diabetes are known high risk factors for developing cholesterol gallstone disease (CGD). CGD is more prevalent in people who consume western diets.[1] Gallstones are formed in the gallbladder when a super saturation of bile cholesterol precipitates as crystals. In the presence of mucin, cholesterol crystals nucleate and grow to form gallstones, which can cause severe abdominal pain and gallbladder inflammation. Currently, the treatment for gallstones is laproscopic cholecystectomy, which is one of the most commonly preformed surgical procedures worldwide. However, cholecystectomy is invasive and can cause surgical complications in terms of morbidity and mortality, and not all patients with symptomatic gallstones are candidates for surgery. Earlier studies have provided evidence for dissolution of cholesterol gallstones through the use of ursodeoxycholic acid (UDCA),[2] which is the only bile acid drug that has been approved by the US FDA for treating liver diseases, including primary biliary cirrhosis and CGD.[3]

UDCA (Figure 1) is a naturally occurring bile acid, derived from cholesterol. It has been shown to alter lipid, glucose, and bile acid metabolism.[3] Indeed, for centuries, bile acids have played important roles in elucidating the mechanisms of metabolic disorders.[4] However, the mechanism by which UDCA works on cholesterol gallstones is unknown. There is, therefore, an urgent need for research defining the mechanism of UDCA and exploring therapies that may have distinct mechanisms of action from past therapies. Our preliminary and recently published data indicate that the farnesoid X receptor (FXR, NR1H4) plays a role in protecting against the development of gallstone disases.[5] Consequently, ligands that bind and activate FXR may be potential therapeutic agents. FXR is a bile acid nuclear receptor that is highly expressed in the liver and gut.[6] Studies from several laboratories have demonstrated FXR serves as a key metabolic integrator that regulates cholesterol, triglyceride, and glucose homeostasis.[7] Bile acids are endogenous signals that can act as FXR ligands [8] and have been shown to reduce adiposity and plasma triglycerides as well as to promote insulin sensitization.[9] The bile acid chenodeoxycholic acid (CDCA) (Figure 1) is one of the most potent endogenous agonists for FXR (EC_{50} ~ 13 μM). However, CDCA is poorly effective as a therapeutic agent because of its toxicity and its promiscuity in activating the intestine expressed G-protein-coupled receptor TGR5, a G-protein-coupled bile acid receptor that mediates cellular responses to bile acids.[10] In contrast, the hydrophilic bile acid UDCA is a widely used therapeutic agent that has minimal side effects.[11]

The available data suggest that UDCA can treat and protect against gallstones, but its molecular target is unknown. Recent studies indicated that UDCA could be working

through a nuclear receptor, the glucocorticoid receptor (GR), but these studies did not show that UDCA directly binds to GR.[11] Others have shown that UDCA binds to FXR expressed in CHOK1 cells, and UDCA may regulate constitutively expressed IBABP (ileal bile acid binding protein) in Caco-2 cells. This suggests that UDCA acts as a partial agonist through an FXR-mediated mechanism.[12] Because activation of FXR through its ligands could control the formation of gallstones,[13] it follows that UDCA may have a key role in treating gallstones with distinct molecular signatures via FXR. However, UDCA is a weak modulator of FXR and has limited clinical efficacy, which is not an ideal clinical tool for testing the FXR-mediated mechanism. There is therefore a need to develop more potent and higher affinity small molecules that can act as targeted therapeutic agents for the treatment of CGD. Building upon the successful synthesis strategy for 6-ECDCA (an ethyl at C-6 on the CDCA ring B),[14] we designed and synthesized 6-αethyl-ursodeoxycholic acid (6EUDCA), an α-ethylated derivative of UDCA. We then identified 6EUDCA as a potent, non-genomic FXR activator that binds FXR stronger than UDCA and shows excellent properties for development as a potential treatment for CGD and related metabolic disorders.

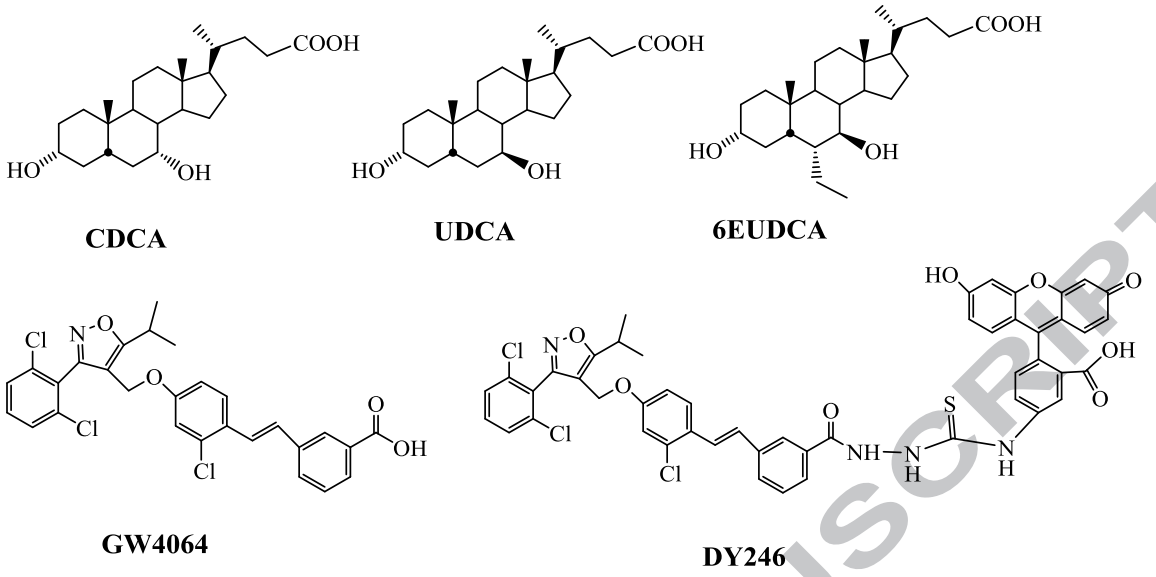

Figure 1. Chemical structures of the naturally-occurring bile acids CDCA and UDCA, as well as the previously disclosed FXR synthetic agonists, 6EUDCA, GW4064, and DY246.

2. Results and discussion

2.1. Chemistry

Previously, we developed an improved synthetic route for large scale production of 6ECDCA (6-αethyl-chenodeoxycholic acid) using the cost-effective and readily-available starting material CDCA.[15] We noted the possibility of using an improved strategy for the synthesis of 6EUDCA from CDCA, which could serve as the key starting material for the production of kilogram quantities of 6EUDCA. 6EUDCA is a novel derivative of UDCA whose preparation was reported in a 2011 U.S. patent application.[16] However, that synthesis methodology was complicated, involving multiple steps, and resulted in a low yield.

To convert CDCA to 6EUDCA, we first evaluated the synthetic procedures for UDCA. The existing methodologies for synthetic UDCA start from the major component of bile acids, cholic acid (CA).[17] In 1954, Kanajawa reported the first synthetic method for the preparation of UDCA from CA in 7-steps (<9% yield).[18] This procedure is still used for the industrial production of UDCA. Several similar synthetic methods starting from CA, including the radical reduction in the last step with an alkaline metal (Na or K) in refluxing alcohol, have been reported recently.[19-21] However, it is difficult to control these vigorous reaction conditions, and the use of concentrated metal bases under high pressure and temperature can easily lead to explosions. With this in mind, several modifications have been reported. In 1980, Faba reported a UDCA synthesis that begins with CA. CA was converted to a methyl ester intermediate and oxidized by Jones reagent with the final step using Zn-EtOH to reduce 7-keto to get UDCA.[22] In 1982, Iida and Chang reported an improved synthetic method that described the four possible 3, 7-dihydroxy acids.[23] This strategy involved a key step for reducing 12-oxo tosylhydrazones. In addition, in 1981, Calzda reported a 6-step synthesis for producing UDCA starting from CA; this method used aluminum alkoxide ($Al(O\textit{i}Pr)_3/\textit{i}PrOH$) instead of alkaline metal in the final reduction step.[24] However, these procedures for producing UDCA and its analogs are long, costly, and complicated and have low yields.

To simplify the production of UDCA, we describe here an improved facile synthesis of UDCA from the readily available starting material CDCA (Figure 2). Key synthetic intermediate **1** was converted from commercially available CDCA according to our previously reported synthetic approach.[15] In this synthetic step, not surprisingly, partially regioselective oxidation proved to be very challenging; the desired corresponding

intermediate **1** from CDCA with pyridinium chlorochromate (PCC) was obtained with 82% yield. Thus, the regioselective installation of carbonyl functionality in the 7-position of CDCA was well done. In contrast to the current regioselective oxidation work, earlier work included a partial oxidation of CDCA,[25] a laborious potassium chromate procedure and a modified synthetic route via CA.[26] These methods were complicated and restricted, clearly suggesting the current regioselective oxidation methodology we improved is facile to carry out for the large scale production of UDCA.

The only chemical difference between UDCA and CDCA is the orientation of the hydroxyl group at the C7 position. In UDCA, the hydroxyl is oriented equatorially (7β-OH), and in CDCA, the hydroxyl is oriented axially (7α-OH). To achieve a 7β-OH at the C7 position, intermediate **1** in Figure 2 was converted to UDCA by Meerwein-Ponndorf-Verley (MPV) type reduction.[27] In particular, by modification of published methods,[11] intermediate **1** was reduced stereoselectively by Al-catalytic reduction with aluminum alkoxide ($Al(O\textit{i}Pr)_3/\textit{i}PrOH$), giving the expected UDCA with the equatorially oriented hydroxyl (7β-OH) at an 82% yield that was highly reproducible. Thus, UDCA was successfully synthesized in 2 steps from CDCA by an improved stereoselective procedure giving a 60-64 % overall yield.

Prompted by production of UDCA from CDCA, we directed our efforts to the synthesis of 6EUDCA from CDCA. The synthesis of the protected 3-hydroxyl of **2** started with the previously reported 6ECDCA preparation.[15] A critical step in the synthesis of 6EUDCA employed selective MPV reduction. In initial experiments, intermediate **2** was treated with base LDA, followed by deprotection with PPTS.[28] This gave compound **3** in a highly efficient manner. We note that a more efficient way to insert an ethyl in the C6

position of CDCA was recently described.[29] However, those procedures for producing an ethyl insertion in the C6 position of CDCA are long and costly, even though they give higher yields.

It is worth noting that the configuration at C-6 on intermediate **3,** the 6α-ethyl derivative, was the only compound formed, this is consistent with previous research by Pellicciari et al.[6] MPV selective reduction of compound **3** with aluminum alkoxide (Al(O*i*Pr)$_3$/*i*PrOH) in the presence of isopropanol led to the formation of 6EUDCA. The stereoselective reduction on substrate **4** is in agreement with the traditional MPV mechanism, which typically yields the more thermodynamically stable, equatorial alcohol product.[27] Thus, our 4-step conversion of CDCA to 6EUDCA was achieved with approximately 15% overall yield (Figure 2).

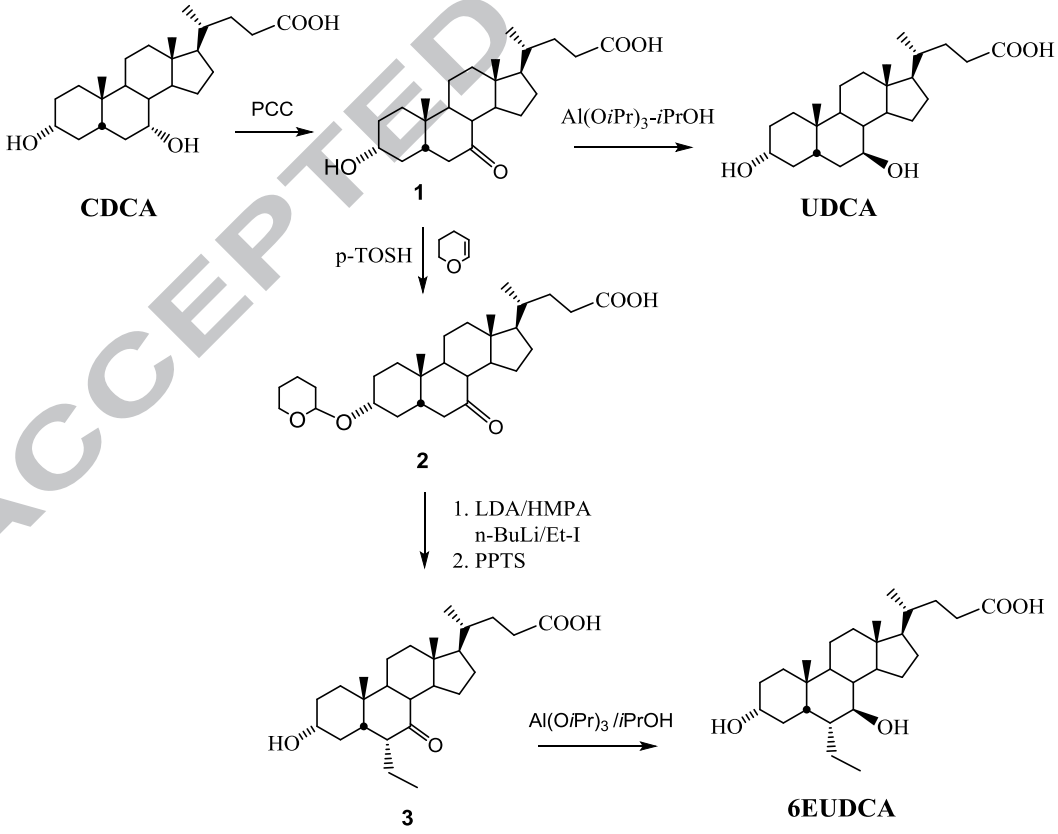

Figure 2. Highly stereoselective synthesis of UDCA and 6EUDCA from CDCA.

The determination of the C7 stereochemistry of 6EUDCA was made by ^{1}H NMR using $CDCl_3$ as a solvent. 6EUDCA and 6ECDCA are epimers, differing only in the configuration of the chiral carbon at C7.[30] The C3 and C7 methine proton peaks of 6EUDCA are easily identified. H7 in 6EUDCA is a multiplet at about 3.36 ppm (J = 8.18 Hz) in $CDCl_3$ whereas the corresponding signal in 6ECDCA is a singlet at about 3.70 ppm.

2.2. Biological evaluations

2.2.1. Time-resolved fluorescence resonance energy transfer binding assay (TR-FRET)

Next, we compared the binding affinities of synthesized 6EUDCA and UDCA to FXR using a time-resolved fluorescence resonance energy transfer binding assay (TR-FRET). Positive and negative controls are described in Experimental Information. Both UDCA and 6EUDCA bound to FXR; however, UDCA bound FXR with a very low affinity and only at high concentrations. In contrast, 6EUDCA bound FXR with higher affinity as indicated by the ability of low concentrations of ligand to produce signals in the TR-FRET assay (Figure 3). These data suggest that 6EUDCA may be a better therapeutic ligand for FXR as compared to UDCA. Additional TR-FRET studies are required to explore and confirm this suggestion.

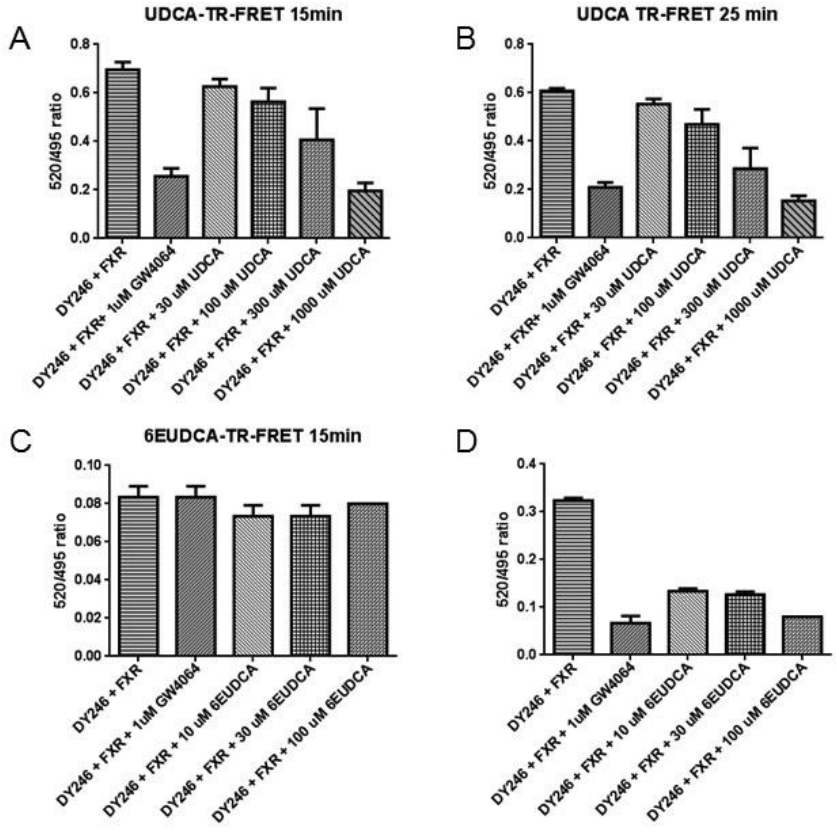

Figure 3. Binding of UDCA and 6EUDCA to FXR in a TR-FRET assay. (A and B) UDCA at high concentrations binds to FXR as indicated by the reduction in fluorescence signal of DY246[31] Shown are data from 15 min (A) and 25 min (B) incubations. 6EUDCA binds strongly to FXR at concentrations as low as 10 μM, as indicated by reduced fluorescence signal of DY246 (C, 15 min incubation and D 25 min incubation). Apparently, 6EUDCA is more potent in binding and activating FXR and has greatly increased potency relative to UDCA. DY246 was chosen as a positive control in the TR-FRET assay because it is a derivative of GW4064, which is a potent FXR agonist.[32] In addition, DY246 is a potent FXR agonist itself, with an EC_{50} of 550 nM and has

successfully been used as a fluorescent probe in a high throughput screening campaign to identify FXR antagonists.[30]

2.2.2. Biological effects of UDCA and 6EUDCA on the human liver cell line HepG2 and on primary mouse hepatocytes

Next, we investigated the biological effects of UDCA and 6EUDCA on the human liver cell line HepG2 and on primary mouse hepatocytes. Initially, we tested whether UDCA or 6EUDCA could affect FXR in HepG2 cells. Our qPCR data indicate that neither UDCA nor 6EUDCA could induce expression of the FXR target gene SHP (Figure 4). GW4064, a known agonist of FXR, was used as a positive control in all our experiments.

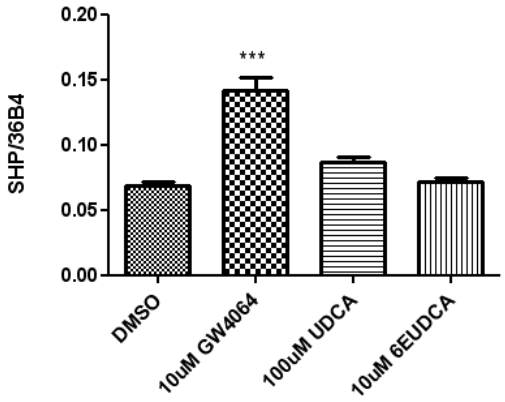

Figure 4. QPCR analysis of the FXR target gene SHP in liver cells. Serum starved HepG2 cells were treated either with DMSO, 10 μM GW4046, 100 μM UDCA, or 10 μM 6EUDCA overnight. Changes in the expression of the SHP gene were analyzed and normalized with the internal standard 36b4. Data are presented as mean ± S.D. ANOVA and Dunnett's multiple comparison tests were applied to determine the significance of the

differences between DMSO and FXR ligands. Asterisks (***) indicate a significant difference in SHP expression ($p<0.0001$) compared to DMSO.

2.2.3. Effect of UDCA and 6EUDCA on AKT and its downstream target GSK3beta

The UDCA result is consistent with previous literature suggesting that UDCA is not an agonist for FXR. Subsequently, based on previous studies in our laboratory, we tested the effect of UDCA and 6EUDCA on AKT and its downstream target GSK3beta. Serum starved HepG2 cells were treated with or without UDCA or 6EUDCA for 10 min. Proteins in cell extracts were separated by SDS-PAGE, and Western blot analysis was performed using specific antibodies against phosphorylated AKT and phosphorylated GSK3beta. Both UDCA and 6EUDCA were effective in inducing AKT phosphorylation in HepG2 cells (Figure 5).

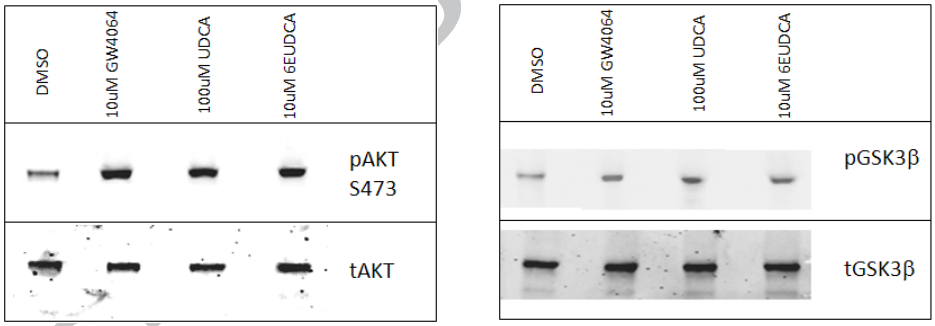

Figure 5. UDCA and 6EUDCA can induce phosphorylation of AKT in liver cells. Serum-starved HepG2 cell extracts were prepared after incubation with DMSO, GW4064, UDCA, or 6EUDCA for 10 min. Extracts were analyzed by Western blot using specific antibodies against pAKT S473 (left panel) or pGSK3beta (right panel).

To further investigate the dose dependence of AKT phosphorylation, we treated HepG2 cells with increasing concentrations of 6EUDCA. Western blot analysis indicated that changes brought about in phosphorylation levels of AKT by 6EUDCA were dose dependent (Figure 6).

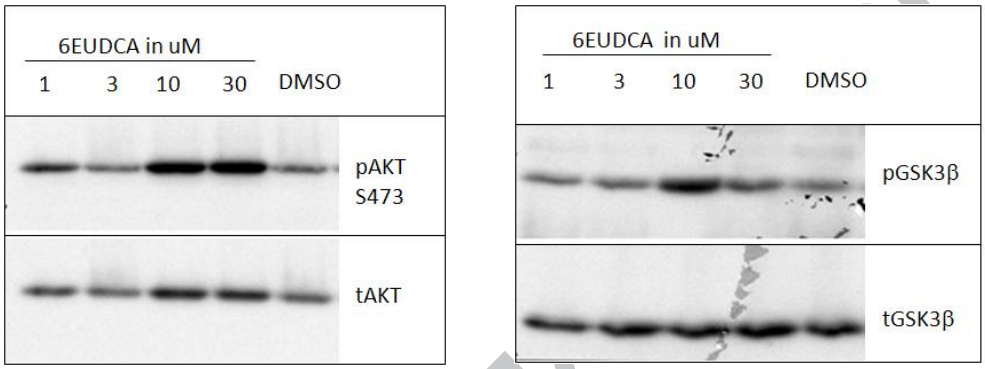

Figure 6. Induction of phosphorylation of AKT by 6EUDCA is concentration dependent in liver cells. Serum-starved HepG2 cell extracts were prepared after incubation with various concentrations of 6EUDCA (DMSO/0 μM, 1 μM, 3 μM, 10 μM, or 30 μM) for 10 min. Extracts were analyzed by Western blot using specific antibodies against pAKT S473 (left panel) or pGSK3beta (right panel). This data shows that much lower concentrations of 6EUDCA were required to induce AKT phosphorylation as compared to UDCA indicating a higher potency for 6EUDCA.

We next tested if UDCA- and 6EUDCA-dependent phosphorylation of AKT could be mediated by FXR. Serum-starved mouse primary hepatocytes isolated from either wild-type or *fxr*$^{-/-}$ mice were treated with or without UDCA or 6EUDCA for 10 min. Cell

extracts were prepared and immunoblotted for phosphorylated AKT or phosphorylated GSK3beta. Western blot analysis indicated that UDCA and 6EUDCA induced phosphorylation of AKT in wild-type hepatocytes but not in *fxr*-/- hepatocytes (Figure 7).

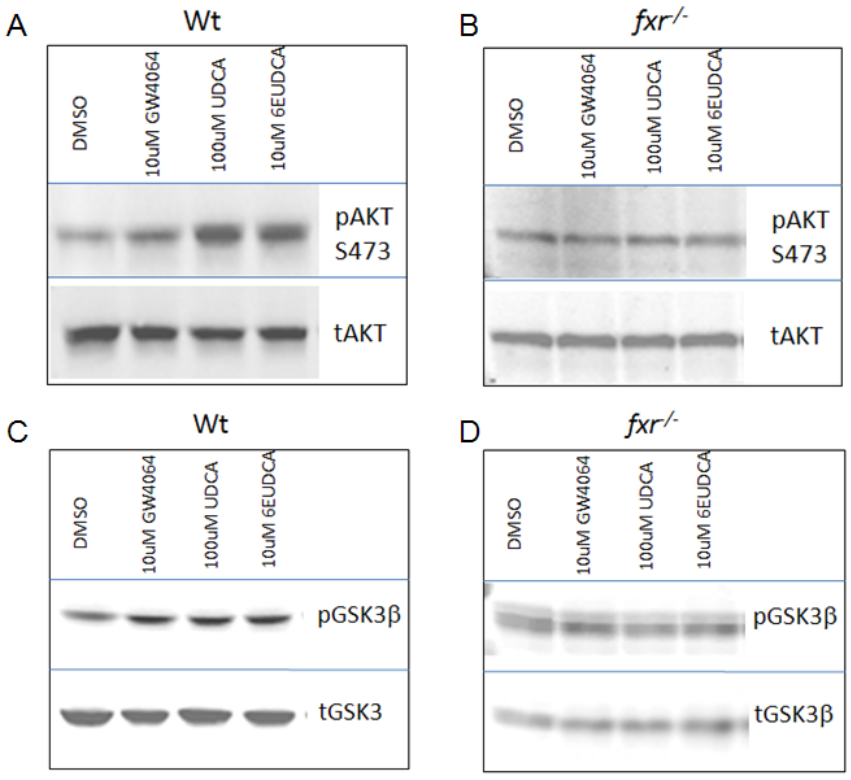

Figure 7. Phosphorylation of AKT and GSK beta is mediated by FXR in liver cells. Serum-starved primary hepatocyte extracts from either wild-type or *fxr*$^{-/-}$ mice were prepared after incubation with FXR ligands (10 min). Extracts were analyzed by Western blot using specific antibodies against pAKT S473 (A and B) or pGSK3beta (C and D). This result strongly suggests that UDCA and 6EUDCA act via FXR in liver cells to induce phosphorylation of AKT. This result also suggests a new non-genomic function for FXR apart from its role as a transcription factor in liver cells.

2.3. Computational modeling of FXR and binding of UDCA and 6EUDCA to FXR

Finally, to explore the binding mode of 6EUDCA and UDCA to FXR, our in-house All-Around-Docking (AAD) methodology [33] was used to predict the best docking site and docking pose. AAD uses Glide software (Schördinger) and allows a small molecule to search the whole surface of a target protein for the docking site with the lowest docking score. The structure of the FXR protein bound to GW4064 was used as the docking target (PDB ID: 3DCT). [34] Using this approach, 6EUDCA, UDCA, and GW4064 were predicted to share the same FXR binding pocket (Figure 8A and 8B), which is formed by M265, M290, R331, H447, W469, *etc.* 6EUDCA contacts more closely with the surface of FXR than that of UDCA. For example, there are 10 residues within 2Å of 6EUDCA; these residues are M265, A291, H294, V325, M328, F329, R331, S332, H447, and W454. In contrast, there are only 3 residues (A291, R331 and S332) within 2Å of UDCA.
Our computational modes show that 6EUDCA forms a larger network of hydrogen bonds with FXR as compared to UDCA. As depicted in Figure 8C, two hydrogen bonds are formed between the UDCA molecule and FXR protein. On the other hand, eight hydrogen bonds are formed between 6EUDCA and FXR (Figure 8D). Furthermore, the 6EUDCA ethyl can form a strong hydrophobic interaction with the W454 residue (Figure 8D). The calculated binding energies predict that FXR associates more strongly with GW4064 (estimated -17.19 kcal/mol binding energy) than UDCA (estimated -9.24 kcal/mol) or 6EUDCA (-10.85 kcal/mol). Based on these results, 6EUDCA is predicted to bind more strongly than UDCA.

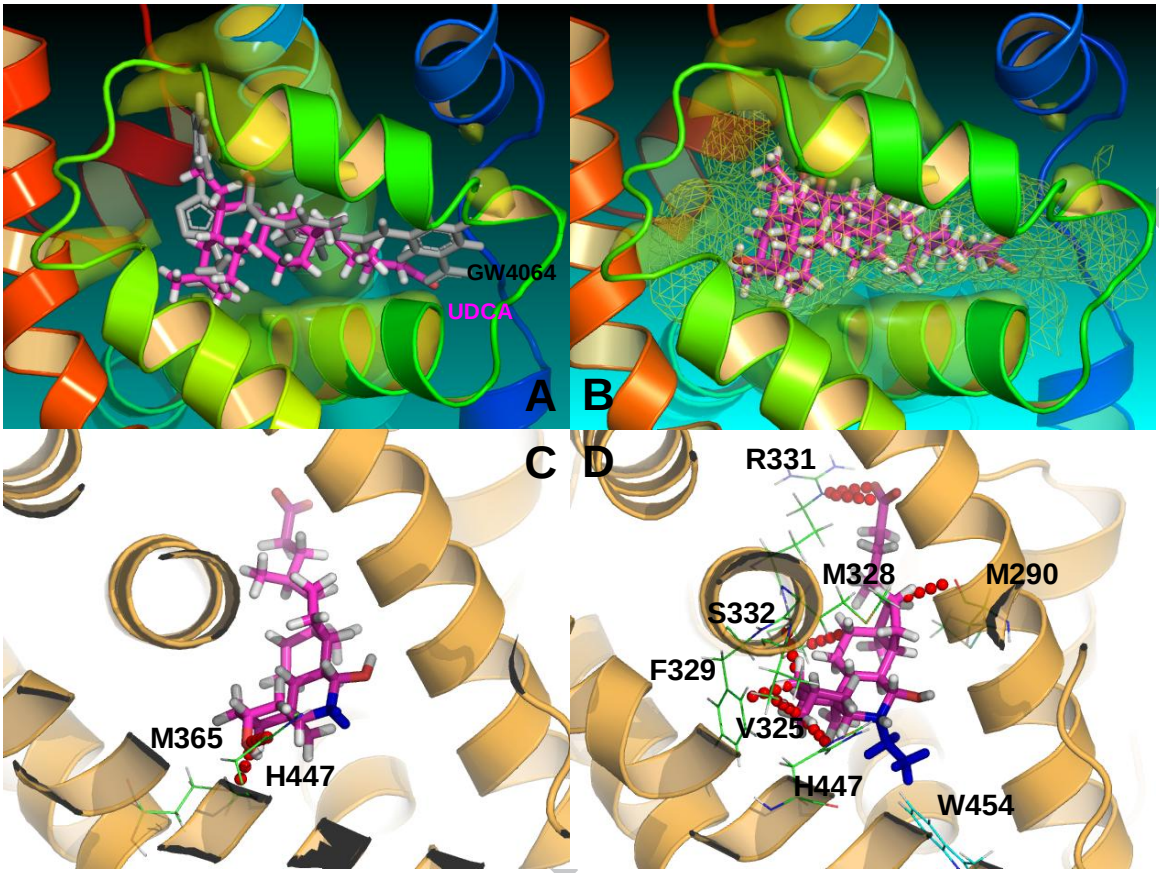

Figure 8. Binding modes of 6EUDCA and UDCA to protein FXR. **A,** All-Around Docking poses of UDCA (pink sticks) and GW4064 molecule (grey sticks) on FXR. **B,** Binding pocket of FXR bound by 6EUDCA. All three compounds above are predicted to bind in the same pocket. **C,** Two hydrogen bonds (red dots) are formed between UDCA and residues H447 and M365 on FXR. The UDCA carbon atom that corresponds to 6EUDCA ethyl is shown in blue. **D,** Eight hydrogen bonds (red dots) are formed between 6EUDCA and residues S332, R331, V325, F329, M328, H447, and M290. The 6EUDCA ethyl is shown in blue and has strong hydrophobic interactions with the W454 residue.

3. Conclusions

In summary, we have described a new chemical synthesis of a modified derivative of UDCA, 6EUDCA, and characterized its biologic effects. As expected based on previous results, neither compound was able to induce expression of the well known FXR target gene SHP. However, both compounds are apparently able to induce increased phosphorylation of AKT and GSK3beta in HepG2 cells, and in primary wild type mouse hepatocytes, but not those from FXR null mice, suggesting a role for UDCA and 6EUDCA, in modulating cell signaling in an FXR-dependent manner. This may lead to the identification of a novel mechanism by which bile acids regulate cell function, and 6EUDCA may be an effective targeted CGD therapeutic. Detailed investigation of the *in vivo* biological effects of 6EUDCA in mouse model of CGD is under way.

4. Experimental section

4.1. Chemistry

Organic reagents were purchased from commercial suppliers unless otherwise noted and were used without further purification. All solvents were analytical or reagent grade. All reactions were carried out in flame-dried glassware under argon or nitrogen. Melting points were determined and reported automatically by an optoelectronic sensor in open capillary tubes and were uncorrected. ^{1}H NMR and ^{13}C NMR spectra were measured at 500 MHz and 125 MHz respectively, and using $CDCl_3$ or CD_3OD as the solvents and tetramethylsilane (Me_4Si) as the internal standard. Flash column chromatography was performed using Sigma-Aldrich silica gel 60 (200-400 mesh), carried out under moderate pressure by using columns of an appropriate size packed and eluted with appropriate

eluents. Silica gel chromatography was performed on a Biotage flash column gradient pump system using 15 cm long columns. All reactions were monitored by TLC on precoated plates (silica gel HLF). TLC spots were visualized either by exposure to iodine vapors or by irradiation with UV light. Organic solvents were removed in vacuum by rotary evaporator. Elemental analyses were performed by Columbia Analytical Services, Inc. Tucson, Arizona.

4.1.1. 3α-Hydroxy-7-keto-5β-cholan-24-oic acid (1). To a suspension of chenodeoxycholic acid (CDCA, 1.0 g, 2.5 mmol, Sigma-Aldrich) and silica gel (4 g, 200-400 mesh, Aldrich) in anhydrous $CHCl_3$ (2 mL) was added, portion-wise, pyridinium chlorochromate (PCC, 0.81 g, 38 mmol) in 25 mL of CH_2Cl_2, and the reaction mixture was stirred at room temperature for 15-20 min. The mixture was filtered and the filtrate was washed with water (20 mL) and brine (20 mL). The organic layer was dried over Na_2SO_4 and concentrated. The resulting crude oil was purified by flash column chromatography (CH_2Cl_2: MeOH 95:5) to afford **1** as a solid (0.76 g, 78% yield), mp 201.2 ^oC (lit.[22] mp 201.1 ^oC). ^1H NMR (500 MHz, CD_3OD) δ 3.51 (m, 1H), 2.94 (m, 1H), 2.52 (t, 1H), 2.30 (m, 2H), 2.19 (m, 6H), 1.70 (m, 2H), 1.43 (m, 4H), 1.31 (m, 6H), 1.19 (s, 3H), 1.12 (m, 4H), 0.92 (d, 3H), 0.66 (s, 3H). ^{13}C NMR (125 MHz, CD_3OD) δ 212.7, 176.8, 70.1, 54.8, 49.2, 48.9, 47.7, 46.0, 44.9, 43.0, 42.4, 38.9, 36.8, 35.1, 34.9, 33.7, 31.0, 30.6, 29.2, 27.8, 24.3, 22.0, 21.4, 17.3, 10.7. Anal. Calcd for $C_{24}H_{38}O_4$: C, 73.81; H, 9.81. Found: C, 73.50; H, 9.63.

4.1.2. 3α-Tetrahydropyranyloxy-7-keto-5β-cholan-24-oic acid (2). To a solution of **1** (0.50 g, 1.3 mmol) in 16 mL of $CHCl_3$:Cl_2CH_2:Et_2O (1:1:2) were added *p*-toluensulfonic acid (0.06 g, 0.3 mmol) and 3,4-dihydro-2H-pyrane (0.41 g, 4.9 mmol). The reaction

mixture was stirred at room temperature for 60 min. Water (20 mL) was added, and the mixture was extracted with EtOAc (3 x 30 ml); the combined organic layers were washed with brine, dried over Na_2SO_4, and concentrated. The resulting crude oil was purified by flash column chromatography (CH_2Cl_2:Et_2O 1:2) to afford **2** as a white solid (0.47 g, 76% yield), mp 160.8 °C (lit.[6] mp 157-159 °C). Selected ^{1}H NMR (600 MHz, $CDCl_3$) δ 4.73 (d, 1H), 3.86 (m, 1H), 3.59 (m, 1H), 3.46 (m, 1H), 2.82 (m, 1H), 1.17 (s, 3H), 0.92 (d, 3H), 0.63 (s, 3H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 212.3, 179.8, 96.4, 62.8, 62.1, 19.8, 18.1, 11.4. Anal. Calcd for $C_{29}H_{46}O_5$: C, 73.38; H, 9.77. Found: C, 73.30; H, 9.76.

4.1.3. 3α-Hydroxy-6α-ethyl-7-keto-5β-cholan-24-oic acid (3). To a solution of **2** (0.30 g, 0.63 mmol) in dry THF (20 mL) at -78°C were added dropwise *n*-butyllithium (1.0 mL, 1.6 M solution in hexane, 1.6 mmol), HMPA (0.7 g, 4 mmoL) and LDA (2.0 mL, 1.8 M in THF/heptane/ethylbenzene, 3.6 mmol). The reaction mixture was stirred for 30 min. Iodoethane (2.0 g, 13 mmol) was slowly added and the reaction mixture was allowed to warm overnight to room temperature. After rotary evaporation, water and ether were added and the aqueous layer was acidified with 10% HCl and extracted with EtOAc (5 x 20 mL). The organic layers were washed with brine, dried over Na_2SO_4, and concentrated to give a yellow oil. After a short column (CH_2Cl_2:Et_2O 1:2), the crude semi-solid was dissolved in ethanol (5 mL) and pyridinium *p*-toluenesulfonate (15 mg, 0.06 mmol) was added. The reaction mixture was stirred at 55 °C for 4 h. After rotary evaporation, the product was passed through a short column (CH_2Cl_2:Et_2O 1:2) to obtain **3** as a semi-solid (0.08 g, 30%) that was used in the next step without further purification.

4.1.4. General Method for Meerwein-Ponndorf-Verley (MPV) Type Reduction

To a solution of aluminum foil (30 eq.) in anhydrous isopropanol (80 eq.) was added mercuric chloride (0.15 eq.). The reaction mixture was warmed up to reflux for 9-12 hours under an atmosphere of nitrogen until the aluminum foil completely dissolved into the isopropanol. After the isopropoxide aluminum formed, it was used directly for the reduction of 3α-hydroxy-7-keto-5β-cholan-24-oic acid (**1**) and 3α-hydroxy-6α-ethyl-7-keto-5β-cholan-24-oic acid (**3**). To this solution, 7-keto analog (1 eq.) was added and the reaction mixture was heated to reflux for 3-5 hours under N_2 and checked by TLC. After cooling, the reaction mixture was dissolved in EtOAc (25 mL) and water was added (30 mL), the solution was acidified to pH 2 with 2N hydrochloric acid, and then extracted with EtOAc (3 x 25 mL). The combined organic layers were washed with water and brine, dried (Na_2SO_4), and evaporated under vacuum.

4.1.5. Usodeoxycholic acid (UDCA): yield: 82%.

Crystallization by EtOAc: pet ether 1:1 gave a white crystal, mp 202.5 ^oC (203-204 °C (lit. Sigma)). Selected ^1H NMR (600 MHz, CD_3OD) δ 3.49 (brs, 2H), 2.37 (m, 1H), 2.22 (m, 1H), 1.93 (m, 2H), 1.87 (m, 3H), 0.97 (s, 3H), 0.96 (d, 3H), 0.71 (s, 3H). Selected ^{13}C NMR (125 MHz, CD_3OD) δ 176.7, 70.7, 70.5, 56.1, 55.1, 20.9, 17.5, 11.0. Anal. Calcd for $C_{24}H_{40}O_4$: C, 73.43; H, 10.27. Found: C, 73.29; H, 9.94.

4.1.6. 3α, 7β-Dihydroxy-6α-ethyl-5β-cholan-24-oic acid (6EUDCA):

Yield: 80%. Solidification by MeOH/CH_2Cl_2/pet ether gave a beige powder, mp 109.5 ^oC. Selected ^1H NMR (600 MHz, CDCl3): δ 3.50 (m, 1H), 3.36 (m, 1H, J = 8.18), 2.25 (m, 1H), 2.15 (m, 1H), 0.91 (d, 3H), 0.88 (s, 3H), 0.77 (t, 3H), 0.64 (s, 3H). ^{13}C NMR (125 MHz, CDCl3): δ 178.4, 75.2, 71.9, 55.8, 54.8, 43.7, 42.8, 41.2, 40.2, 39.6, 35.5,

34.4, 30.8, 30.7, 30.5, 28.3, 27.0, 23.5, 21.4, 20.8, 18.3, 12.0, 11.8. Anal. Calcd for $C_{26}H_{44}O_4$: C, 74.24; H, 10.54. Found: C, 74.00; H, 10.01.

4.2. Biology

4.2.1. Antibodies

Antibodies for pAKT S473, pGSK3beta, total AKT and total GSK3beta used for western blot analysis were obtained from Cell Signaling (USA).

4.2.2. Cell culture

HepG2 cells were purchased from American Type Culture Collection. The cells were cultured in Minimal Essential Medium (MEM) (Cellgro, Manassas, VA) supplemented with 100 U/ml penicillin/streptomycin sulfate (Mediatech, Herndon, VA) and 10% (v/v) heat-inactivated fetal bovine serum (Irvine Scientific, Santa Ana, CA). Primary hepatocytes were isolated from mouse liver tissues and cultured in Dulbecco's modified Eagle medium and F-12 (Cellgro, Manassas, VA) supplemented with 100U/ml penicillin G/streptomycin and 10% (v/v) heat-inactivated fetal bovine serum. Cells were cultured in insulin or serum free media for 16 hr before performing all experiments unless otherwise mentioned. Primary mouse hepatocytes were isolated as described from C57BL6/J mice by using collagenase type IV; 5×10^5 cells per well were plated in six-well collagen-coated plates and cultured in DMEM/Ham's F12 media.[35]

4.2.3. TR-FRET assay

To assess the binding of UDCA and 6EUDCA, we performed TR-FRET assay using DY246 as an hFXR fluorescent probe as described earlier.[30] For this assay, 30 nl of 10 mM testing chemical was transferred with a pintool into 15 μL of a mixture of GST–

hFXR and Tb-anti-GST in a 384-well black plate, and then 5 μL of 40 nM DY246 was dispensed to give a final volume of 20 μL/well with 10 nM GST–hFXR, 1.5 nM Tb-anti-GST, UDCA (1000 μM, 300 μM, or 100 μM) or 6EUDCA (100 μM, 30 μM, or 10μM) and 10 nM DY246. In addition, selected wells containing 5 μM GW4064 or DMSO were used as positive and negative controls, respectively. The final DMSO concentration was 0.23% in all wells. The plates were then spun down after a brief shake and incubated for either 15 or 25 min at room temperature. The TR-FRET signal was then collected for each well with an Envison plate reader using an excitation wavelength of 340 nm and emission wavelengths of 520 nm and 490 nm.

4.2.4. RNA isolation and Quantitative Real-time PCR

RNA was isolation, reverse transcription reactions, and real time PCR were performed as described previously.[36] SHP primers forward 5'-GCC CTG CAC TCT CGC TTT CT-3'; reverse 5'-CAA CTG GGC ACC GAG GCA ACA GTT G-3'were used to amplify using Sybr green master mix purchased from applied biosystems (Foster City, CA). Amplification of 36b4 with specific primers forward 5'-TGG AGT CTT TCT GGA GCC TT-3'; reverse 5'-TCC TGT TGC AGG TGT GCG AT-3' was used as internal control. Absolute mRNA expression was quantified using standard curve method. All primers used were Sybr green-based synthesized by IDT. Sybr green was purchased from Applied Biosystems (Foster City, CA). Amplification of 36b4 was used as an internal control. Absolute mRNA expression was quantified using the standard curve method.

4.2.5. Western blot analysis

Western blot analysis was performed according to methods described earlier.[37] The antibodies used are listed above (4.2.1). Briefly, serum-starved HepG2 cells or mouse

primary hepatocytes from flox/flox or *fxr*-/- mice cultured in collagen-coated 6-well culture dishes were used to make total cell lysates. Proteins were resolved on SDS-PAGE gels and transferred onto nitrocellulose membrane and blotted with antibodies against phosphor AKT (S473) and pGSK3beta. The signal with secondary antibody was detected with either an Odyssey 3.0 infrared scanner or ECL chemiluminiscence.

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Graphical Abstract