Structure-activity relationship of bile acids and bile acid analogs in regard to FXR activation

Tomofumi Fujino,* Mizuho Une,[1,†] Tsuneo Imanaka,[§] Kazuhide Inoue,* and Tomoko Nishimaki-Mogami*

Division of Biosignaling,* National Institute of Health Sciences, Tokyo, Japan; Division of Medicinal Chemistry Programs for Pharmaceutical Sciences,[†] Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima, Japan; and Department of Biological Chemistry,[§] Faculty of Pharmaceutical Sciences, Toyama Medical and Pharmaceutical University, Toyama, Japan

Abstract **The farnesoid X receptor (FXR) is a bile acid-activated nuclear receptor that plays a major role in bile acid and cholesterol metabolism. To obtain an insight into the structure-activity relationships of FXR ligands, we investigated the functional roles of structural elements in the physiological ligands chenodeoxycholic acid [CDCA; (3α,7α)], cholic acid [CA; (3α,7α,12α)], deoxycholic acid [DCA; (3α,12α)], and lithocholic acid (3α) in regard to FXR activation in a cell-based FXR response element-driven luciferase assay and an in vitro coactivator association assay. Conversion of the carboxyl group of CDCA or CA to an alcohol did not greatly diminish their ability to activate FXR. In contrast, the 7β-epimers of the alcohols were inactive, indicating that the bile alcohols retained the ligand properties of the original bile acids and that the 7β-hydroxyl group diminished their FXR-activating effect. Similarly, hydroxyl epimers of DCA exhibited decreased activity compared with DCA, indicating a negative effect of 3β- or 12β-hydroxyl groups. Introduction of an alkyl group at the 7β- or 3β-position of CDCA resulted in diminished FXR activation in the following order of alkyl groups: 7-ethyl = 7-propyl > 3-methyl > 7-methyl. These results indicate that bulky substituents, whether hydroxyl groups or alkyl residues, at the β-position of cholanoids decrease their ability to activate FXR.—** Fujino, T., M. Une, T. Imanaka, K. Inoue, and T. Nishimaki-Mogami. **Structure-activity relationship of bile acids and bile acid analogs in regard to FXR activation. *J. Lipid Res.* 2004.** 45: **132–138.**

Supplementary key words bile alcohols • steroid receptor coactivator-1 • cholesterol 7α-hydroxylase • CV-1 cell • farnesoid X receptor

Bile acid biosynthesis is a major component of cholesterol homeostasis and represents an important pathway for the elimination of cholesterol from the body. Cholesterol 7α-hydroxylase (CYP7A1), a rate-limiting enzyme for bile acid formation, is negatively controlled by bile acids returning to the liver (1–3). Recent studies have shown that this downregulation is mediated by the farnesoid X receptor (FXR), a nuclear receptor activated by several hydrophobic bile acids (4–7). Activation of FXR induces an orphan nuclear receptor, the small heterodimer partner (SHP), which forms a complex with liver receptor homolog-1 (LRH-1). Because LRH-1 is required for transcription of CYP7A1, increased SHP levels result in decreased CYP7A1 expression (8, 9). Sterol 12α-hydroxylase (CYP8B1), which catalyzes the synthesis of cholic acid (CA), is also repressed by bile acids through an FXR-dependent mechanism (10, 11). In addition, FXR is expressed in the intestine, where bile acids are efficiently reabsorbed. The intestinal bile acid binding protein, a cytosolic bile acid transporter in ileal enterocytes, is induced by bile acids that activate FXR (12). Thus, FXR is considered to play a critical role in cholesterol and bile acid metabolism.

Several compounds not structurally related to bile acids have been shown to activate or antagonize FXR (13–15). Although chenodeoxycholic acid (CDCA) is the most potent physiological ligand of FXR, its 7β-epimer, ursodeoxycholic acid (UDCA), does not activate FXR and has no effect on CYP7A1 in vivo (3, 5, 6). Thus, it is likely that the minor structural modifications of bile acid lead to significant changes in their ability to activate FXR.

Enhancement of bile acid biosynthesis by releasing it from the negative feedback control is considered to be a strategy for developing hypocholesterolemic agents. Sev-

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Abbreviations: CA, cholic acid (3α,7α,12α-trihydroxy-5β-cholanoic acid); CDCA, chenodeoxycholic acid (3α,7α-dihydroxy-5β-cholanoic acid); CYP7A1, cholesterol 7α-hydroxylase; CYP8B1, sterol 12α-hydroxylase; DCA, deoxycholic acid (3α,12α-dihydroxy-5β-cholanoic acid); FXR, farnesoid X receptor; LCA, lithocholic acid (3α-hydroxy-5β-cholanoic acid); LRH-1, liver receptor homolog-1; RXR, retinoid X receptor; SHP, small heterodimer partner; SRC-1, steroid receptor coactivator-1; UCA, ursocholic acid (3α,7β,12α-trihydroxy-5β-cholanoic acid); UDCA, ursodeoxycholic acid (3α,7β-dihydroxy-5β-cholanoic acid).

[1] To whom correspondence should be addressed.
e-mail: mune@hiroshima-u.ac.jp

eral bile acid analogs have already been developed as hypocholesterolemic agents (16–19), and elucidation of the structure-activity relationships of FXR ligands should provide a useful strategy for the development of new therapeutic agents.

The present study focused on the relationship between the activity and structure of the nucleus or the side chain and was performed to elucidate the ability of naturally occurring bile acids, synthetic bile acid analogs, and bile alcohols (depicted in **Fig. 1**) to activate FXR in a cell-based luciferase assay and a coactivator association assay. The latter was a ligand sensor assay in vitro in which ligand-dependent recruitment of the steroid receptor coactivator-1 (SRC-1) to FXR was monitored by surface plasmon resonance (SPR) technology. Bile alcohols derived from bile acids were shown to exhibit ligand properties for FXR comparable to those of the original bile acids. In addition, introduction of a hydroxyl or alkyl substituent into the β-site of steroid nuclei decreased their ability to activate FXR, probably by diminishing binding affinity to the ligand binding domain (LBD) of FXR.

MATERIALS AND METHODS

Cholanoids

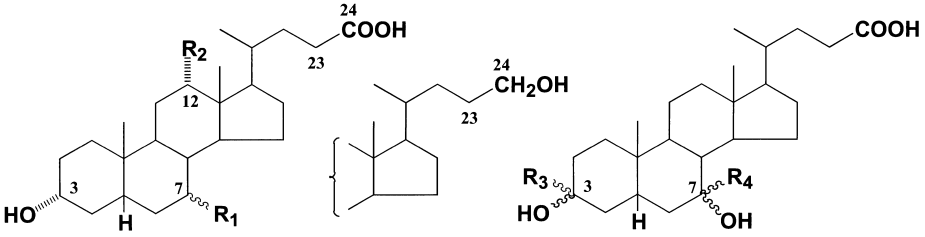
CA, CDCA, deoxycholic acid (DCA), UDCA, and lithocholic acid (LCA) were commercial products. Ursocholic acid was prepared from CA as described previously (20). 3α,7α-Dihydroxy-7β-methyl-5β-cholanoic acid, 3α,7α-dihydroxy-7β-ethyl-5β-cholanoic acid, 3α,7α-dihydroxy-7β-propyl-5β-cholanoic acid, and 3α,7β-dihydroxy-7α-methyl-5β-cholanoic acid were prepared as described previously (21, 22). 5β-Cholane-3α,7β,12α,24-tetrol, 5β-cholane-3α,7α,12α,24-tetrol, 5β-cholane-3α,7α,24-triol, and 5β-cholane-3α,7β,24-triol were prepared from the corresponding bile acids as described previously (23). 3β-Hydroxy-5β-cholanoic acid and 3β,12α-, 3α,12β-, and 3β,12β-dihydroxy-5β-cholanoic acids were prepared by the method described previously (24).

Synthesis of 3α,7α-dihydroxy-3β-methyl-5β-cholanoic acid and 3β,7α-dihydroxy-3α-methyl-5β-cholanoic acid

7α-Hydroxy-3-oxo-5β-cholanoic acid was prepared from CDCA by treatment with aluminum *t*-butoxide according to the method described previously (24). To a solution of the 3-oxo-compound dissolved in 100 ml of dry benzene was added drop-wise with stirring a 3.0 M ethereal solution of methyl magnesium iodide. The reaction mixture was stirred at room temperature for 2 h, diluted with ice-cold water, and extracted with ethyl ether after acidification with 1 N HCl. The extract was washed with water to neutrality, dried over anhydrous Na_2SO_4, and evaporated to dryness. The mixture of free acids was treated with diazomethane, and the resulting methyl ester derivatives were placed on a column of silica gel (70 g; silica gel G, 35-70 mesh; Merck) and eluted with benzene-ethyl acetate mixtures. The 3β-hydroxy isomer and 3α-hydroxy isomer were eluted with 10% ethyl acetate in benzene and 20% ethyl acetate in benzene, respectively. Alkaline hydrolysis of the two isomers afforded the corresponding free acids 3β,7α-dihydroxy-3α-methyl-5β-cholanoic acid (120 mg), melting point 215–218°C (from ethyl acetate), proton nuclear magnetic resonance (δ-ppm): 0.73 (3H, s, 18-CH_3), 1.02 (3H, d, J = 5.4 Hz, 21-CH_3), 1.10 (3H, s, 19-CH_3), 1.39 (3H, s, 3α-CH_3), 4.06 (1H, m, 7β-H); and 3α,7α-dihydroxy-3β-methyl-5β-cholanoic acid (240 mg), melting point 107–111°C (from ethyl acetate), proton nuclear magnetic resonance (δ-ppm): 0.71 (3H, s, 18-CH_3), 1.00 (3H, s, 19-CH_3), 1.00 (3H, d, J = 5.4 Hz, 21-CH_3), 1.47 (3H, s, 3β-CH_3), 4.04 (1H, m, 7β-H).

Cell culture and preparation of the FXR LBD

CV-1 cells were obtained from the Japanese Collection of Research Bioresources Cell Bank (Tokyo, Japan) and maintained in DMEM containing 10% fetal calf serum. Human FXR LBD was expressed in *Escherichia coli* as a glutathione *S*-transferase fusion protein and purified on glutathione beads after cleaving with precision protease.



Bile acids (I-VI) / The corresponding Alcohols (Ia-IVa)

	R_1	R_2
I Cholic acid	OH(α)	OH
II Ursocholic acid	OH(β)	OH
III Chenodeoxycholic acid	OH(α)	H
IV Ursodeoxycholic acid	OH(β)	H
V Deoxycholic acid	H	OH
VI Lithocholic acid	H	H

Alkylated Chenodeoxycholic acid

	R_3	R_4
3β-Me-CDCA	CH_3(β)	H(β)
7β-Me-CDCA	H(β)	CH_3(β)
7β-Et-CDCA	H(β)	C_2H_5(β)
7β-Pr-CDCA	H(β)	C_3H_7(β)
7α-Me-UDCA	H(β)	CH_3(α)
3α-Me-isoCDCA	CH_3(α)	H(β)

Fig. 1. Structures of bile acids and bile acid analogs. CDCA, chenodeoxycholic acid; 7β-Et-CDCA, 3α,7α-dihydroxy-7β-ethyl-5β-cholanoic acid; 3α-Me-isoCDCA, 3β,7α-dihydroxy-3α-methyl-5β-cholanoic acid; 3β-Me-CDCA, 3α,7α-dihydroxy-3β-methyl-5β-cholanoic acid; 7α-Me-UDCA, 3α,7β-dihydroxy-7α-methyl-5β-cholanoic acid; 7β-Me-CDCA, 3α,7α-dihydroxy-7β-methyl-5β-cholanoic acid; 7β-Pr-CDCA, 3α,7α-dihydroxy-7β-propyl-5β-cholanoic acid; Ia, 5β-cholane-3α,7α,12α,24-tetrol; IIa, 5β-cholane-3α,7β,12α,24-tetrol; IIIa, 5β-cholane-3α,7α,24-triol; IVa, 5β-cholane-3α,7β,24-triol.

Plasmid constructs

An FXR response element (FXRE)-driven luciferase reporter plasmid (pFXRE-tk-Luc) was constructed by inserting complementary oligonucleotides containing four copies of FXRE from the phospholipid transfer protein promoter (5′-aaactgaGGGTCAgTGACCCaagtgaa-3′) (25) and overhangs for *Kpn*I and *Bgl*II upstream of the thymidine kinase (tk) promoter. The expression vectors pcDNA3.1-FXR and pcDNA3.1-RXRα were constructed by inserting the cDNAs encoding full-length human FXR and human retinoid X receptor-α (RXRα), respectively, into mammalian expression vector pcDNA3.1 (Invitrogen, Carlsbad, CA).

Transient transfections and reporter gene assays

CV-1 cells were cotransfected with 187.5 ng of pFXRE-tk-Luc, 62.5 ng each of pcDNA3.1-FXR and pcDNA3.1-RXRα, and 187.5 ng of pSV-β-galactosidase control vector (Promega, Madison, WI) with PolyFect (Qiagen, Chatsworth, CA) when cells reached 80% confluence in 24-well plates. Three hours after transfection, cells were exposed to bile acids at concentrations of 0 to 100 μM for 24 h. Cell extracts were prepared with the cell lysis buffer (Promega), and the luciferase and β-galactosidase activities were determined. Luciferase activity was normalized to that of β-galactosidase in each sample.

Coactivator association assay using SPR

The measurements were performed using a BIAcore 3000 (BIAcore AB, Uppsala, Sweden) as described elsewhere (T. Fujino, Y. Sato, M. Une, Y. Kanayasu-Toyoda, T. Yamaguchi, K. Shudo, K. Inoue, and T. Nishimaki-Mogami, unpublished observations). Briefly, wild-type peptide from human SRC-1 (CPSSHSSLTARHKILHRLLQEGSPS-$CONH_2$) containing the nuclear receptor interaction consensus motif (LXXLL) and the consensus-mutated peptide (CPSSHSSLTARHKIAHRALQEGSPS-$CONH_2$) were biotinylated and immobilized on individual surfaces of streptavidin chips to responses of 122 resonance units (RU) and 124 RU, respectively. FXR LBD (1–4 μM) preincubated with ligands for 1 h was injected over the surfaces in a running buffer composed of 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.05% Tween 20, and 0.5% DMSO at 25°C. After completion of the injection (120 s), the complex formed was washed with buffer for an additional 120 s. The chip surfaces were regenerated down to the peptide level by subsequent application of a 30 s pulse of 0.1% SDS and 10 mM NaOH. Kinetic parameters [association rate constant (k_a), dissociation rate constant (k_d), and equilibrium dissociation constant (K_d)] were determined by nonlinear regression analysis using PRISM software (GraphPad Software Inc., San Diego, CA). The BIAcore system allows simultaneous detection of the interaction events in four flow cells. To eliminate responses attributable to nonspecific interactions, sensorgrams detected in wild-type SRC1-immobilized cells were routinely corrected with sensorgrams from cells with mutant SRC1.

RESULTS

Bile alcohols derived from CDCA and CA activate FXR

CDCA has been shown to be the most potent natural ligand of FXR (5–7). We investigated whether conversion of the carboxyl group to an alcohol affects the ability of CDCA to activate FXR. In a transient transfection assay using an FXRE-driven luciferase construct and human FXR and RXR expression plasmids, 5β-cholane-3α,7α,24-triol (CDC-OH), a bile alcohol derived from CDCA, exhibited activity comparable to that of CDCA (**Fig. 2A**). Furthermore, in a coactivator association assay using SPR in vitro, CDC-OH caused potent interaction between FXR and SRC-1 peptide (Fig. 2B), indicating that CDC-OH is a bona fide ligand for FXR. Kinetic analysis showed that CDC-OH and CDCA decreased the K_d by 5-fold and 13-fold, respectively, compared with the no-ligand control

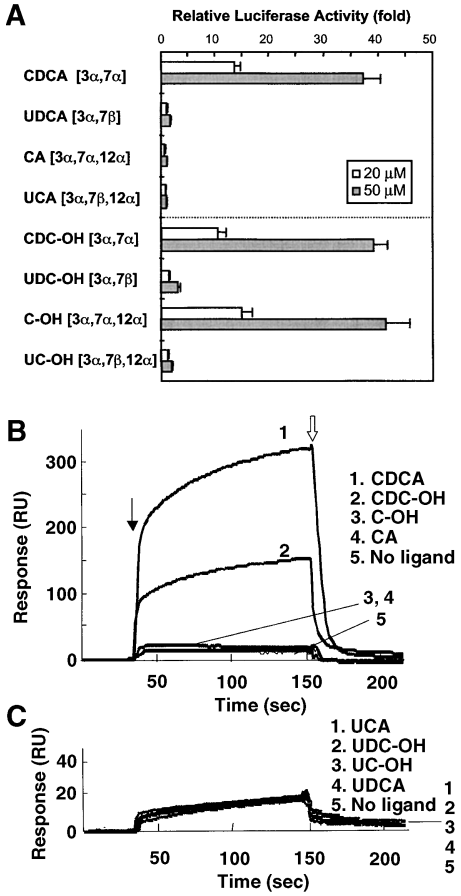


Fig. 2. Effect of bile acids and their corresponding alcohols on farnesoid X receptor (FXR) activation in a cell-based luciferase assay (A) and in a coactivator recruitment assay using surface plasmon resonance (B and C). A: CV-1 cells were cotransfected with an FXR response element-driven luciferase reporter plasmid and expression vectors for FXR and retinoid X receptor together with a pSV-β-galactosidase control vector. Cells were exposed to vehicle alone or to 20 or 50 μM bile acids, as indicated. Luciferase activity was normalized by using β-galactosidase as an internal control. Data are expressed as multiples of induction relative to vehicle-treated cells and represent means ± SD of three determinations. B and C: FXR ligand binding domain (LBD) (4 μM) preincubated with vehicle alone or 100 μM bile acids for 1 h was injected over the sensorchip surface immobilized with steroid receptor coactivator-1 (SRC-1) peptide. The sensorgrams shown were corrected for the use of a surface immobilized with a mutant peptide. The arrow and the open arrow indicate the beginning and end of the injections, respectively. CA, cholic acid; CDC-OH, 5β-cholane-3α,7α,24-triol; C-OH, 5β-cholestane-3α,7α,12α,24-tetrol; DCA, deoxycholic acid; LCA, lithocholic acid; UCA, ursocholic acid; UC-OH, 5β-cholane-3α,7β,12α,24-tetrol; UDCA, ursodeoxycholic acid; UDC-OH, 5β-cholane-3α,7β,24-triol; RU, resonance units. The positions and stereochemistry of the hydroxyl groups on the ring structure are indicated.

(**Table 1**), indicating increased affinity. Thus, the data clearly show that conversion of the carboxyl group in CDCA to an alcohol marginally affected its ability to activate FXR.

CA was inactive in our assay system using CV-1 cells (Fig. 2A). CA has been shown to activate FXR in a cell-based assay if the transporter is expressed (6, 7). In contrast, 5β-cholestane-3α,7α,12α,24-tetrol (C-OH), a bile alcohol prepared from CA, efficiently activated FXR (Fig. 2A), indicating that the conversion of the carboxyl group to an alcohol facilitated its transport into cells instead of abolishing the ability of CA to activate FXR. This neutral compound, C-OH, appears to be transported into cells by passive diffusion. Although C-OH, as well as CA, was inactive in the coactivator association assay (Fig. 2B), the inability of CA to promote the SRC-1/FXR association in vitro has been observed in a fluorescence resonance energy transfer (FRET) assay (5, 6).

7β-Isomers of CDC-OH and C-OH do not activate FXR

UDCA, a 7β-epimer of CDCA, has been shown not to activate FXR (5–7). To identify the role of the 7β-hydroxyl group in the diminished ligand potency for FXR, we prepared bile alcohols from UDCA and ursocholic acid (UCA), the 7β-epimer of CA. 5β-Cholane-3α,7β,24-triol (UDC-OH), the 7β-epimer of CDC-OH, was inactive as the original UDCA in both the cell-based luciferase assay (Fig. 2A) and the coactivator association assay in vitro (Fig. 2C). Similarly, 5β-cholane-3α,7β,12α,24-tetrol (UC-OH), the 7β-epimer of C-OH, was inactive in regard to FXR transactivation (Fig. 2A). These observations clearly show that the stereochemistry of the hydroxyl group at the C-7 position is critical for FXR activation, irrespective of whether the side chain is an acid or an alcohol.

3β-Hydroxyl or 12β-hydroxyl groups diminish the effect on FXR activation

To further elucidate the role of the orientation of the hydroxyl groups at C-3 and C-12 in FXR activation, we tested a series of epimers of DCA by the cell-based luciferase reporter assay. The 3β-epimer and the 12β-epimer of DCA each had less than half the activity of DCA (**Fig. 3A**). Similarly, the activity of the 3β-epimer of LCA accounted for only 30% of the activity of LCA (Fig. 3B). Thus, epimerization of the 3β- and/or 12β-hydroxyl groups in DCA and LCA diminished their FXR-activating effects.

Alkyl substituent at the 7β- or 3β-position of CDCA diminishes its ability to activate FXR

To gain insight into the diminishing effect of the 7β- or 3β-hydroxyl group on FXR activation, we examined the effect of alkyl substituents at the 7β- or 3β-position of CDCA. As shown in **Fig. 4A**, introduction of a methyl group at the C-7β position of CDCA decreased its ability to activate FXR by 65–80% of that of CDCA. The presence of a bulkier substituent (ethyl- or propyl-group) reduced its activity even more (to 5–10% and 10–20% of that of CDCA, respectively). In contrast, methyl substitution at the 7α-position of UDCA had little effect on its activity (Fig. 4B). Introduction of a methyl group at the C-3β position in CDCA diminished the effect of CDCA by 70–80% (Fig. 4B), whereas the activity of its 3α-methyl counterpart, 3β,7α-dihydroxy-3α-methyl-5β-cholanoic acid, was reduced only slightly compared with that of CDCA (Fig. 4B).

TABLE 1. Affinity and rate constants for farnesoid X receptor/steroid receptor coactivator-1 interactions induced by various bile acids and bile acid derivatives

Ligand	Association Rate Constant	Dissociation Rate Constant	Equilibrium Dissociation Constant
	$M^{-1}\ s^{-1}$	s^{-1}	μM
No ligand	0.51×10^4	2.14×10^{-1}	42.0
CDCA	4.76×10^4	1.59×10^{-1}	3.34
CA	0.99×10^4	3.63×10^{-1}	36.7
CDC-OH	3.92×10^4	3.39×10^{-1}	8.65
C-OH	0.92×10^4	3.50×10^{-1}	38.0
7β-Me-CDCA	2.45×10^4	3.02×10^{-1}	12.3
3β-Me-CDCA	1.55×10^4	3.34×10^{-1}	21.5

CA, cholic acid; CDCA, chenodeoxycholic acid; CDC-OH, 5β-cholane-3α,7α,24-triol; C-OH, 5β-cholestane-3α,7α,12α,24-tetrol; 3β-Me-CDCA, 3α,7α-dihydroxy-3β-methyl-5β-cholanoic acid; 7β-Me-CDCA, 3α,7α-dihydroxy-7β-methyl-5β-cholanoic acid.

The ability of CDCA to induce SRC-1/FXR interaction was reduced by the introduction of an alkyl substituent either at the 3β or the 7β-position of CDCA (Fig. 4C). Sensorgrams (Fig. 4C) and kinetic parameters (Table 1) revealed the following order of β-alkyl groups accounting to their ability to diminish the activity of CDCA: 7-ethyl = 7-propyl > 3-methyl > 7-methyl; this order was identical to the order for FXR transactivation in the luciferase assay (Fig. 4A). Thus, the data clearly show that alkyl substituents at either the 7β- or the 3β-position diminish the ability of CDCA to activate FXR.

We then further tested the possibility that these β-alkylated CDCAs act as FXR antagonists. However, the β-alkylated CDCAs (100 μM) had no effect on the SRC-1/FXR interaction elicited by 20 μM CDCA (Fig. 4D).

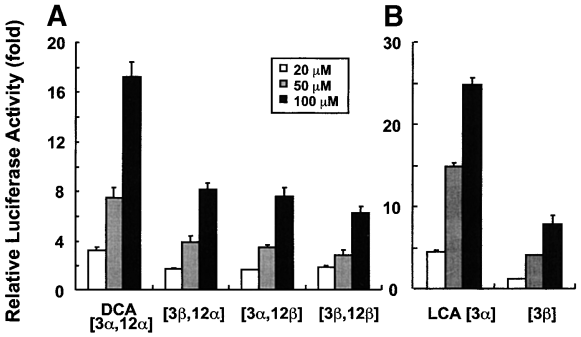


Fig. 3. Effect of hydroxyl epimers of DCA and LCA on FXR activation. FXR activation was evaluated by the luciferase reporter assay as described in the legend to Fig. 2. DCA [3α,12α], 3α,12α-dihydroxy-5β-cholanoic acid; [3α,12β], 3α,12β-dihydroxy-5β-cholanoic acid; [3β,12α], 3β,12α-dihydroxy-5β-cholanoic acid; [3β,12β], 3β,12β-dihydroxy-5β-cholanoic acid; LCA [3α], 3α-hydroxy-5β-cholanoic acid; [3β], 3β-hydroxy-5β-cholanoic acid. Data are expressed as multiples of induction relative to vehicle-treated cells and represent means ± SD of three determinations.

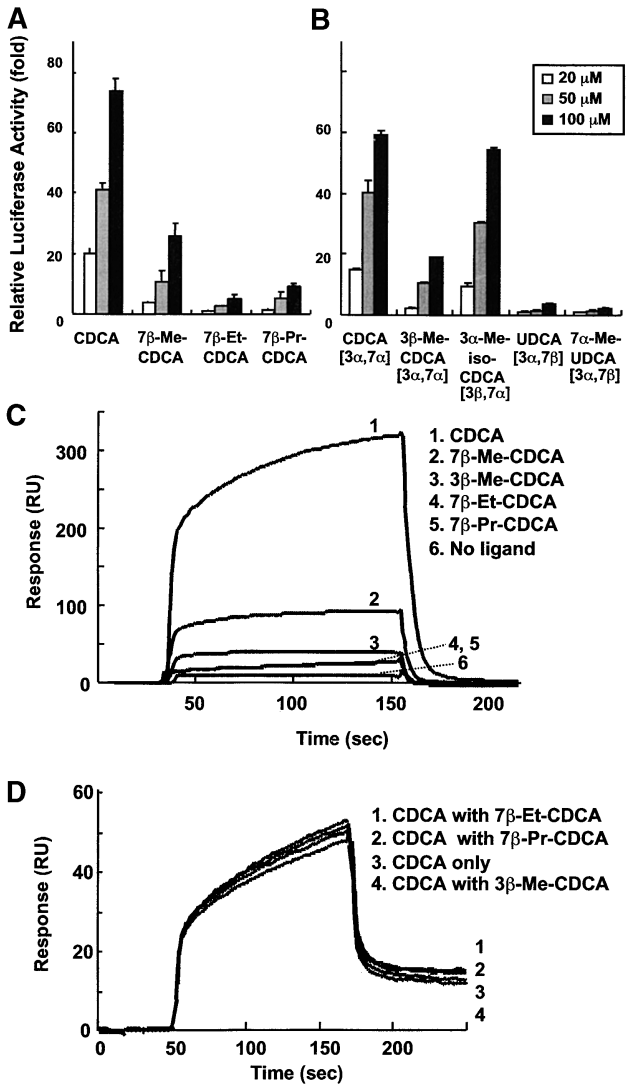


Fig. 4. Effect of alkylated CDCA analogs on FXR activation in the cell-based luciferase assay (A, B) and the coactivator recruitment assay (C, D). FXR activation was evaluated by the luciferase reporter assay (A, B) and the coactivator recruitment assay (C) as described in the legend to Fig. 2. D: FXR LBD preincubated with either vehicle or 100 μM alkylated CDCA together with 20 μM CDCA for 1 h was injected over the surface of the SRC-1 peptide-immobilized sensorchip. The positions and stereochemistry of the hydroxyl groups on the ring structure are indicated. Data are expressed as multiples of induction relative to vehicle-treated cells and represent means ± SD of three determinations.

DISCUSSION

The present study was carried out to investigate the relationship between the structural aspects of bile acids and their FXR-activating activities by modifying the structure of the physiological ligands CDCA, CA, DCA, and LCA.

We showed that bile acid-derived bile alcohols still exhibit activities and ligand properties in relation to FXR comparable to those of the original bile acids. CDCA-derived alcohol (CDC-OH) is a potent activator of FXR that is as efficient as CDCA in both the cell-based luciferase assay (Fig. 2A) and the coactivator association assay in vitro (Fig. 2B). Similarly, C-OH prepared from CA potently activated FXR in the cell-based luciferase assay (Fig. 2A). The discrepancy between the results obtained from the cell-based reporter assay and the in vitro coactivator association assay is still obscure. However, it should be mentioned that CA was always inactive in the coactivator association assay in vitro, regardless of the detection method [FRET (5, 6) or SPR in Fig. 2B]. Because CA is capable of promoting SRC-1/FXR association in a cell-based mammalian two-hybrid assay if the transporter is expressed (26), the activation of FXR by CA may require unknown factor(s) other than SRC-1. Thus, our data clearly indicate that the conversion of the carboxyl group of bile acids to an alcohol only modestly affects their binding affinity and activation efficacy. Conjugation of bile acids with glycine or taurine has been shown to have little effect on FXR activation (5–7). Based on the results of the crystal structure study, the carbonyl oxygen at C-24 in both conjugated and unconjugated bile acids is predicted to form the hydrogen bond with Arg328 in the FXR LBD (27). The binding affinity of CDC-OH or C-OH also may be preserved through the hydrogen bonding of the alcoholic oxygen at C-24 with Arg328.

In contrast to CDC-OH and C-OH, their 7β-epimers, alcohols derived from UDCA and UCA, were still as inactive as the original bile acids (Fig. 2A), indicating that cholanoids with 7β-hydroxyl substituents do not activate FXR. Based on the finding that UDCA, the 7β-epimer of CDCA, is inactive (5–7), it has been suggested that the stereochemistry of the hydroxyl group at C-7 in the steroid nucleus is critical for FXR activation. Our data also strongly support this assumption.

We also demonstrated the importance of the orientation of the hydroxyl group at the C-3 and C-12 positions. The negative effect of 3β- or 12β-hydroxyl groups was shown in experiments using epimers of DCA and LCA (Fig. 3). These results may explain the previous finding that an epimerization of DCA derepressed the expression of CYP7A mRNA (3).

Differences in physiological activity and physicochemical properties between CDCA and UDCA are usually explained by their hydrophobic and hydrophilic balance. The 7β-hydroxyl group in UDCA reduces the hydrophobic area (β-site) of the cholanoic acid nucleus, whereas the 7α-hydroxyl group, together with the 3α-hydroxyl group, constitutes the hydrophilic area of CDCA. Thus, it is possible that the decreased hydrophobicity at the β-site results in the diminished ligand potency of UDCA. However, in the present study, we showed that the introduction of a nonpolar (hydrophobic) alkyl group at the 7β-position decreased the ability of CDCA to activate FXR in the luciferase assay (Fig. 4A) and to promote coactivator association (Fig. 4C) to the level of UDCA, even though the hydrophobicity of the β-site of the steroid ring was increased by this modification. Furthermore, the reduced ability to activate FXR as a result of the methyl substitution was amplified by ethyl and propyl substitutions. Similar findings were observed when a methyl group was introduced at the C-3β position. These findings indicate that

steric hindrance attributable to a bulky substituent in the C-7β or C-3β position, not a reduction in the hydrophobic area of the β-site, is the cause of the diminished ligand potency. The inability of β-alkylated CDCAs to compete with CDCA for coactivator recruitment (Fig. 4D) suggests that a bulky substituent at the C-7β or C-3β position of CDCA decreases its binding affinity for the FXR ligand binding pocket. A structural study predicted that UDCA having a 7β-hydroxyl group would create an open ligand binding pocket, resulting in the partial inhibition of coactivator association (28). Thus, it is likely that an alkyl substituent at the C-7β position would have a similar steric effect, leading to decreased coactivator association. The decreased FXR activation by alkylated CDCAs provides a mechanism for our previous finding that 7β-alkylated CDCA represses CYP7A activity in primary cultured rat hepatocytes less efficiently than does CDCA (16).

In contrast to the negative effect of 7β-methyl substitution in CDCA, the introduction of a methyl group at the C-7α position of UDCA did not affect its activity. It is likely that the methyl group at the C-7α position is unable to increase the affinity to FXR, whereas the 7α-hydroxyl group in CDCA forms hydrogen bonds with Tyr366 in the FXR LBD (27, 28). An alternative explanation is that the decreased affinity caused by steric hindrance by the 7β-hydroxyl group is too great to identify the effect of the 7α-methyl group. This appears to be in contrast to ethyl substitution at the C-6α position of CDCA, which leads to great enhancement of FXR ligand potency (29).

Although the 3β-hydroxyl group in DCA and LCA (Fig. 3A, B) had a negative effect, 3β,7α-dihydroxy-3α-methyl-5β-cholanoic acid exhibited activity comparable to that of CDCA (Fig. 3B), which seems to suggest that the 3α-methyl group increases its affinity. A crystal structure study with 3-deoxy-CDCA demonstrated that the 3α-hydroxyl group of CDCA is not responsible for FXR activation (27).

In conclusion, this study demonstrated that the steroid nucleus structure of bile acids was critical for FXR activation. Substituents, such as an alkyl group or a hydroxyl group at the β-site of the steroid nucleus in bile acids, diminished ligand potency. Conversion of the carboxyl groups of bile acids to alcohols did not affect ligand properties but markedly facilitated transport into cells. These findings provide basic information that will be useful in the design of new drugs to improve cholesterol/bile acid homeostasis.

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REFERENCES

1. Pandak, W. M., Z. R. Vlahcevic, D. M. Heuman, K. S. Redford, J. Y. Chiang, and P. B. Hylemon. 1994. Effects of different bile salts on steady-state mRNA levels and transcriptional activity of cholesterol 7α-hydroxylase. *Hepatology.* **19:** 941–947.
2. Russell, D. W., and K. D. Setchell. 1992. Bile acid biosynthesis. *Biochemistry.* **31:** 4737–4749.
3. Stravitz, R. T., P. B. Hylemon, D. M. Heuman, L. R. Hagey, C. D. Schteingart, H. T. Ton-Nu, A. F. Hofmann, and Z. R. Vlahcevic. 1993. Transcriptional regulation of cholesterol 7α-hydroxylase mRNA by conjugated bile acids in primary cultures of rat hepatocytes. *J. Biol. Chem.* **268:** 13987–13993.
4. Chiang, J. Y., R. Kimmel, C. Weinberger, and D. Stroup. 2000. Farnesoid X receptor responds to bile acids and represses cholesterol 7α-hydroxylase gene (CYP7A1) transcription. *J. Biol. Chem.* **275:** 10918–10924.
5. Makishima, M., A. Y. Okamoto, J. J. Repa, H. Tu, R. M. Learned, A. Luk, M. V. Hull, K. D. Lustig, D. J. Mangelsdorf, and B. Shan. 1999. Identification of a nuclear receptor for bile acids. *Science.* **284:** 1362–1365.
6. Parks, D. J., S. G. Blanchard, R. K. Bledsoe, G. Chandra, T. G. Consler, S. A. Kliewer, J. B. Stimmel, T. M. Willson, A. M. Zavacki, D. D. Moore, and J. M. Lehmann. 1999. Bile acids: natural ligands for an orphan nuclear receptor. *Science.* **284:** 1365–1368.
7. Wang, H., J. Chen, K. Hollister, L. C. Sowers, and B. M. Forman. 1999. Endogenous bile acids are ligands for the nuclear receptor FXR/BAR. *Mol. Cell.* **3:** 543–553.
8. Goodwin, B., S. A. Jones, R. R. Price, M. A. Watson, D. D. McKee, L. B. Moore, C. Galardi, J. G. Wilson, M. C. Lewis, M. E. Roth, P. R. Maloney, T. M. Willson, and S. A. Kliewer. 2000. A regulatory cascade of the nuclear receptors FXR, SHP-1, and LRH-1 represses bile acid biosynthesis. *Mol. Cell.* **6:** 517–526.
9. Lu, T. T., M. Makishima, J. J. Repa, K. Schoonjans, T. A. Kerr, J. Auwerx, and D. J. Mangelsdorf. 2000. Molecular basis for feedback regulation of bile acid synthesis by nuclear receptors. *Mol. Cell.* **6:** 507–515.
10. Brendel, C., K. Schoonjans, O. A. Botrugno, E. Treuter, and J. Auwerx. 2002. The small heterodimer partner interacts with the liver X receptor α and represses its transcriptional activity. *Mol. Endocrinol.* **16:** 2065–2076.
11. Castillo-Olivares, A., and G. Gil. 2001. Suppression of sterol 12α-hydroxylase transcription by the short heterodimer partner: insights into the repression mechanism. *Nucleic Acids Res.* **29:** 4035–4042.
12. Kanda, T., L. Foucand, Y. Nakamura, I. Niot, P. Besnard, M. Fujita, Y. Sakai, K. Hatakeyama, T. Ono, and H. Fujii. 1998. Regulation of expression of human intestinal bile acid-binding protein in Caco-2 cells. *Biochem. J.* **330:** 261–265.
13. Howard, W. R., J. A. Pospisil, E. Njolito, and D. J. Noonan. 2000. Catabolites of cholesterol synthesis pathways and forskolin as activators of the farnesoid X-activated nuclear receptor. *Toxicol. Appl. Pharmacol.* **163:** 195–202.
14. Urizar, N. L., A. B. Liverman, D. T. Dodds, F. V. Silva, P. Ordentlich, Y. Yan, F. J. Gonzalez, R. A. Heyman, D. J. Mangelsdorf, and D. D. Moore. 2002. A natural product that lowers cholesterol as an antagonist ligand for FXR. *Science.* **296:** 1703–1706.
15. Zavacki, A. M., J. M. Lehmann, W. Seol, T. M. Willson, S. A. Kliewer, and D. D. Moore. 1997. Activation of the orphan receptor RIP14 by retinoids. *Proc. Natl. Acad. Sci. USA.* **94:** 7909–7914.
16. Kim, H. G., M. Une, A. Hino, H. Wada, M. Yoshii, T. Kuramoto, and K. Fujimura. 2000. Bile acid sulfonate and 7-alkylated bile acid analogs: effect on intestinal absorption of taurocholate and cholesterol 7α-hydroxylase activity in cultured rat hepatocytes. *Steroids.* **65:** 24–28.
17. Kim, H. G., M. Une, T. Kuramoto, M. Noshiro, and K. Fujimura. 2001. Hypocholesterolemic effect of bile acid sulfonate analogs in hamsters. *Biol. Pharm. Bull.* **24:** 218–220.
18. Mikami, T., K. Kihira, S. Ikawa, M. Yoshii, S. Miki, E. H. Mosbach, and T. Hoshita. 1993. Metabolism of sulfonate analogs of ursodeoxycholic acid and their effects on biliary bile acid composition in hamsters. *J. Lipid Res.* **34:** 429–435.
19. Une, M., K. Yamanaga, E. H. Mosbach, K. Tsujimura, and T. Hoshita. 1990. Metabolism of 7β-alkyl chenodeoxycholic acid analogs and their effect on cholesterol metabolism in hamsters. *J. Lipid Res.* **31:** 1015–1021.
20. Batta, A. K., S. K. Aggarwal, G. Salen, and S. Shefer. 1991. Selective reduction of oxo bile acids: synthesis of 3β-, 7β-, and 12β-hydroxy bile acids. *J. Lipid Res.* **32:** 977–983.
21. Une, M., B. I. Cohen, and E. H. Mosbach. 1984. New bile acid analogs: 3α,7α-dihydroxy-7β-methyl-5β-cholanoic acid, 3α,7β-dihydroxy-7α-methyl-5β-cholanoic acid, and 3α-hydroxy-7ξ-methyl-5β-cholanoic acid. *J. Lipid Res.* **25:** 407–410.
22. Une, M., K. Yamanaga, E. H. Mosbach, S. Kuroki, and T. Hoshita. 1989. Synthesis of bile acid analogs: 7-alkylated chenodeoxycholic acids. *Steroids.* **53:** 97–105.

23. Kihira, K., M. Yoshii, A. Okamoto, S. Ikawa, H. Ishii, and T. Hoshita. 1990. Synthesis of new bile salt analogues, sodium 3α,7α-dihydroxy-5β-cholane-24-sulfonate and sodium 3α,7β-dihydroxy-5β-cholane-24-sulfonate. *J. Lipid Res.* **31:** 1323–1326.
24. Danielsson, H., P. Eneroth, K. Hellstrom, and J. Sjovall. 1962. Synthesis of some 3β-hydroxylated bile acids and the isolation of 3β,12α-dihydroxy-5β-cholanic acid from feces. *J. Biol. Chem.* **237:** 3657–3659.
25. Laffitte, B. A., H. R. Kast, C. M. Nguyen, A. M. Zavacki, D. D. Moore, and P. A. Edwards. 2000. Identification of the DNA binding specificity and potential target genes for the farnesoid X-activated receptor. *J. Biol. Chem.* **275:** 10638–10647.
26. Makishima, M., T. T. Lu, W. Xie, G. K. Whitfield, H. Domoto, R. M. Evans, M. R. Haussler, and D. J. Mangelsdorf. 2002. Vitamin D receptor as an intestinal bile acid sensor. *Science.* **296:** 1313–1316.
27. Mi, L. Z., S. Devarakonda, J. M. Harp, Q. Han, R. Pellicciari, T. M. Willson, S. Khorasanizadeh, and F. Rastinejad. 2003. Structural basis for bile acid binding and activation of the nuclear receptor FXR. *Mol. Cell.* **11:** 1093–1100.
28. Downes, M., M. A. Verdecia, A. J. Roecker, R. Hughes, J. B. Hogenesch, H. R. Kast-Woelbern, M. E. Bowman, J. L. Ferrer, A. M. Anisfeld, P. A. Edwards, J. M. Rosenfeld, J. G. Alvarez, J. P. Noel, K. C. Nicolaou, and R. M. Evans. 2003. A chemical, genetic, and structural analysis of the nuclear bile acid receptor FXR. *Mol. Cell.* **11:** 1079–1092.
29. Pellicciari, R., S. Fiorucci, E. Camaioni, C. Clerici, G. Costantino, P. R. Maloney, A. Morelli, D. J. Parks, and T. M. Willson. 2002. 6α-Ethyl-chenodeoxycholic acid (6-ECDCA), a potent and selective FXR agonist endowed with anticholestatic activity. *J. Med. Chem.* **45:** 3569–3572.