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5α-Bile alcohols function as farnesoid X receptor antagonists ☆

Tomoko Nishimaki-Mogami [a,*], Yosuke Kawahara [b], Norimasa Tamehiro [a], Takemi Yoshida [b], Kazuhide Inoue [a], Yasuo Ohno [a], Taku Nagao [a], Mizuho Une [c]

[a] National Institute of Health Sciences, Setagaya-ku, Tokyo 158-8501, Japan
[b] School of Pharmaceutical Sciences, Showa University, Shinagawa-ku, Tokyo 142-8555, Japan
[c] Faculty of Pharmaceutical Sciences, Hiroshima International University, Kure, Hiroshima 737-0112, Japan

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Abstract

The farnesoid X receptor (FXR) is a bile acid/alcohol-activated nuclear receptor that regulates lipid homeostasis. Unlike other steroid receptors, FXR binds bile acids in an orientation that allows the steroid nucleus A ring to face helix 12 in the receptor, a crucial domain for coactivator-recruitment. Because most naturally occurring bile acids and alcohols contain a *cis*-oriented A ring, which is distinct from that of other steroids and cholesterol metabolites, we investigated the role of this 5β-configuration in FXR activation. The results showed that the 5β-(A/B *cis*) bile alcohols 5β-cyprinol and bufol are potent FXR agonists, whereas their 5α-(A/B *trans*) counterparts antagonize FXR transactivation and target gene expression. Both isomers bound to FXR, but their ability to induce coactivator-recruitment and thereby induce transactivation differed. These findings suggest a critical role for the A-ring orientation of bile salts in agonist/antagonist function.

Keywords: Nuclear receptor; Farnesoid X receptor; Bile acid; Bile alcohol; Agonist; Antagonist; Coactivator

The farnesoid X receptor (FXR; NR1H4) is a nuclear receptor that is activated by bile acids [1,2] and bile alcohols [3,4], and it plays an essential role in bile acid/cholesterol homeostasis [5,6]. FXR belongs to the steroid hormone receptor superfamily, however, crystal structure studies have suggested that bile acids bind FXR with their steroid backbone flipped head to tail, the reverse orientation of all other steroid hormones, when they bind to their cognate receptors. Steroid hormones, such as testosterone, glucocorticoids, and estrogen, are oriented with their D rings facing helix 12 of their respective receptors [7–9], whereas the A ring of bile acids faces helix 12 of FXR [10,11].

Helix 12, the most C-terminal helix in the ligand binding domain of nuclear receptors, plays a crucial role in ligand-dependent receptor activation. Binding of an agonist to a receptor leads to a conformational change that allows the receptor to interact with a coactivator, which mediates ligand-dependent transcription of the receptor [12]. In this activated state, helix 12, the activation function 2 (AF2), functions as a molecular switch and forms one side of the coactivator binding pocket [13]. Structural analysis studies have demonstrated that agonist and antagonist bind at the same site within the core of the ligand-binding domain, but induce different conformations [8,10,11,14]. Agonists have been shown to stabilize the agonist conformation of helix 12 via direct or indirect interactions, and partial agonists or antagonists have been shown to destabilize it.

Unlike those of other steroids and cholesterol metabolites, the A rings of most naturally occurring bile acids are *cis*-oriented (5β-configuration). Because structural studies have shown that the A ring of bile acids is in contact with several amino acid residues on helices 11 and 12

☆ *Abbreviations:* FXR, farnesoid X receptor; CDCA, chenodeoxycholic acid; CYP7A1, cholesterol 7α-hydroxylase; SHP, the small heterodimer partner; BSEP, bile salt export pump; PXR, pregnane X receptor.

* Corresponding author. Fax: +81 3 3707 6950.
E-mail address: mogami@nihs.go.jp (T. Nishimaki-Mogami).

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[10,11], it seems likely that the 5β-(A/B *cis*) ring juncture of bile acids plays a critical role in stabilizing the agonist-bound conformation of helix 12.

Bile alcohols are produced as intermediates in the bile acid synthetic pathway in mammals and as end-products of cholesterol catabolism in most evolutionarily primitive vertebrates [15]. We have shown that bile alcohols possess FXR-ligand properties similar to those of the corresponding bile acids [3]. Although the majority of naturally occurring bile alcohols are 5β-bile alcohols, few species of fishes and frogs produce 5α-bile alcohols containing a *trans*-oriented A ring. The bile alcohol 5α-cyprinol was originally isolated from the bile of *Cyprinus carpi* [16], the Asiatic carp, and 5α-bufol was isolated from the bile of lungfish [17] and frogs [18] (Fig. 1). Since our preliminary experiments showed that their 5β-counterparts, 5β-cyprinol and 5β-bufol, are potent agonists of human FXR, in this study we investigated whether these 5α-bile alcohols possess the ability to bind to FXR and recruit a coactivator. The results showed that both of these 5α-bile alcohols are capable of binding to FXR but are unable to induce coactivator-association, and as a result antagonize FXR activation.

FXR activation has been shown to repress the expression of cholesterol 7α-hydroxylase (CYP7A1), a rate-limiting enzyme in the bile acid biosynthetic pathway, by inducing an orphan nuclear receptor, the small heterodimer partner (SHP) [19,20]. FXR also up-regulates expression of the bile salt export pump (BSEP) [21], which represents the major canalicular bile salt export pump of the liver. We also investigated whether the 5α-bile alcohols modulate FXR-target gene expression.

Materials and methods

Bile alcohols and chemicals. Cholic acid and chenodeoxycholic acid were commercial products. 5α- and 5β-Cholestane-3α,7α,12α,26,27-pentols (5α-cyprinol and 5β-cyprinol) were isolated from the bile of carp [16,18]. 5α- and 5β-Cholestane-3α,7α,12α,25,26-pentols (5α-bufol and 5β-bufol) were isolated from the bile of frogs and toads, respectively [18,22]. GW4064 is synthesized according to the published procedures [23].

Transient transfections and reporter gene assays. HepG2 cells were maintained in DMEM containing 10% FCS and 100 μg/ml kanamycin, and they were seeded in 24-well plates 24 h prior to transfection. Cells were transfected with 85 ng pFXRE-tk-Luc [3], 25 ng each of the pcDNA3.1 expression vectors for human FXR (NR1H4) and RXRα, and either 65 ng of *Renilla* luciferase vector (phRL-TK) or pSV-β-galactosidase vector (Promega) with Effectene (Qiagen). Three hours after transfection, cells were exposed for 24 h to bile acids or bile alcohols in the medium containing 0.5% delipidated FBS. Cells were lysed and luciferase activity was determined. Firefly luciferase activity was normalized to *Renilla* luciferase or β-galactosidase activity for each well.

Coactivator-association assay using fluorescence polarization. The assay was performed essentially according to the published procedure [4]. TAMRA-labeled peptide (100 nM, with amino acid sequence ILRKLLQE) was incubated for 1 h with 1.5 μM of purified GST-fused human FXR ligand binding domain (residues 244–472) and ligands in 100 μl of buffer (10 mM Hepes, 150 mM NaCl, 2 mM $MgCl_2$, and 5 mM DTT at pH 7.9) in a black polypropylene 96-well plate on a shaker. Ligand-dependent recruitment of the coactivator peptide was measured as increases in fluorescence polarization with a Mithras LB-940 multilabel reader (Berthold).

mRNA analysis by real-time quantitative RT-PCRs. Gene-specific mRNA quantitation was performed by real-time PCR on an ABI Prism

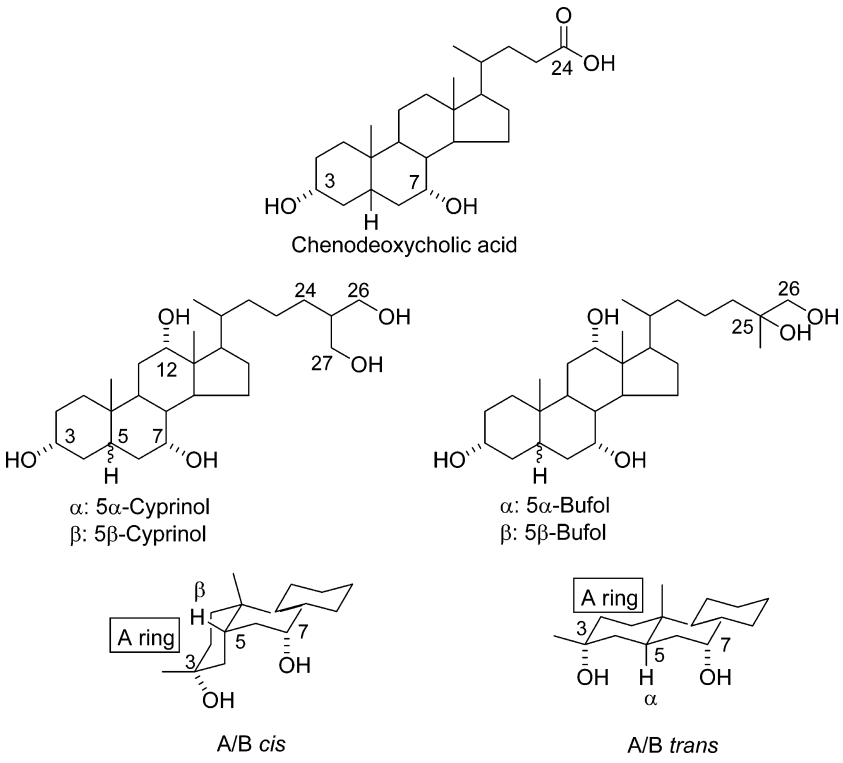

Fig. 1. Structure of bile alcohols and chenodeoxycholic acid (CDCA). 5α- and 5β-Cyprinol, 5α- and 5β-cholestane-3α,7α,12α,26,27-pentols; 5α- and 5β-bufol, 5α- and 5β-cholestane-3α,7α,12α,25,26-pentols; chenodeoxycholic acid, 3α,7α-dihydroxy-5β-cholanoic acid.

7700 sequence detection system (Applied Biosystems). Human hepatoma-derived HepG2 cells were exposed to bile acids or bile alcohols in DMEM containing 0.5% delipidated FBS for 20 h. Total RNA extracted with the RNeasy Mini Kit (Qiagen) was treated with DNAase according to the manufacturer's instructions (Qiagen). The relative expression levels of mRNA were determined using the TaqMan one-step RT-PCR Master Mix Reagent Kit. The primer/probe sequences for human BESP, CYP7A1, and SHP have been reported previously [4,24].

Other methods. Cell viability was checked by leakage of lactate dehydrogenase into the medium. Statistical significance was determined by ANOVA followed by the Student Newman–Keuls method.

Results

The ability of bile alcohols to activate human FXR was assessed by means of a transient transfection assay. HepG2 cells were cotransfected with a FXRE-driven luciferase reporter plasmid and expression plasmids for FXR and RXRα. Exposure of the cells to 5β-cyprinol or 5β-bufol, bile alcohols containing two hydroxyl groups in their side chain (Fig. 1), led to the induction of luciferase activity at levels comparable to that of the most potent physiological FXR ligand, CDCA (Fig. 2A), whereas their 5α-counterparts, 5α-cyprinol and 5α-bufol, had little effect. However, these 5α-bile alcohols inhibited the transactivation elicited by either 50 μM CDCA (Fig. 2B) or 1 μM GW4064, a synthetic FXR agonist structurally unrelated to bile acids [23] (Fig. 2C).

In an in vitro coactivator-recruitment assay, 5β-cyprinol and 5β-bufol induced a dose-dependent interaction of SRC-1 peptide with FXR, but not with LXRα (Fig. 3A). By contrast, 5α-cyprinol induced a very weak interaction, accounting for 20% of 5β-cyprinol-induced interaction (Fig. 3B). 5α-Bufol induced no interaction at all. When assayed with 1 μM GW4064, these 5α-bile alcohols reduced (by 80%) the GW4064-elicited interaction (Fig. 3C). These findings show that 5α-cyprinol and 5α-bufol act as FXR antagonists, whereas their 5β-counterparts, 5β-cyprinol and 5β-bufol, are FXR agonists.

We used real-time quantitative RT-PCR to investigate the effect of these bile alcohols on the expression of FXR-target genes in HepG2 cells. 5β-Cyprinol and 5β-bufol increased the BSEP mRNA level, whereas 5α-cyprinol and 5α-bufol had little effect (Fig. 4A). When combined with 50 μM CDCA, these 5α-bile alcohols decreased CDCA-elicited induction of BSEP mRNA in a dose-dependent manner (Fig. 4B).

5β-Cyprinol and 5β-bufol increased the SHP mRNA level and markedly reduced the CYP7A1 mRNA level (Fig. 4A). The SHP mRNA elevation by 5α-bufol and 5α-cyprinol was small or insignificant. However, unexpectedly, these 5α-bile alcohols markedly repressed CYP7A1 expression (by 90% and 80%, respectively, at 50 μM). By contrast, 90% reduction in the CYP7A1 expression by CDCA was accompanied by a 5.2-fold elevation of the SHP level. When combined with CDCA, these 5α-bile alcohols further enhanced CDCA-elicited repression of CYP7A1, although SHP expression was unchanged or decreased instead (Fig. 4B).

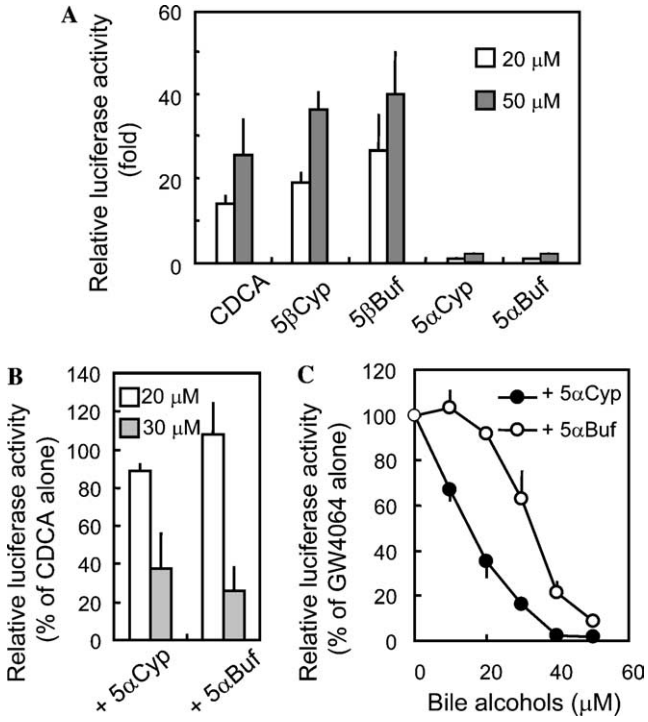

Fig. 2. 5β-Bile alcohols activate FXR, but 5α-bile alcohols function as antagonists in the cellular transactivation assay. (A) HepG2 cells were transfected with expression plasmids for human FXR and RXRα, and the $FXRE_{PLTP}$x4-tk-luc reporter plasmid together with a *Renilla* luciferase plasmid as a control. Cells were exposed to vehicle alone or to 20–50 μM of the bile alcohols indicated. Luciferase activity in the cell extract was normalized to *Renilla* luciferase activity and expressed as fold induction relative to vehicle-exposed cells. The values are means ± SD of three experiments. (B,C) Cells were transfected as in (A), except that β-Gal was used as an internal control, and exposed to 50 μM CDCA (B) or 1 μM GW4064 (C) in the presence of the concentrations of 5α-cyprinol (5α Cyp) or 5α-bufol (5α Buf) indicated. Exposure of cells to CDCA or GW4064 alone caused a 70- or 115-fold induction, respectively, relative to vehicle-exposed cells. The values are the means ± SD of three experiments.

Discussion

Bile acids and bile alcohols are produced as the terminal catabolites of cholesterol, and as amphipathic steroids they also play important roles in intestinal lipid absorption. Their unusual 5β-A/B *cis* ring juncture provides a structure that allows them to function as excellent detergents [5]. In addition, by activating FXR as physiological ligands, bile acids and alcohols directly modulate expression of genes involved in the biosynthesis/catabolism, excretion, and absorption of bile acids and cholesterol [6]. In the present study, we investigated the role of the A/B ring juncture configuration of bile salts in ligand-activation of FXR.

The results of both cell-based and in vitro assays showed that 5β-cyprinol and 5β-bufol, but not their 5α-counterparts, 5α-cyprinol and 5α-bufol, function as potent FXR agonists (Figs. 2A and 3A), indicating that the *cis*-orientation of the A ring is essential for FXR activation. It was noteworthy that, when assayed with CDCA, the 5α-alcohols potently inhibited agonist-elicited FXR transactivation

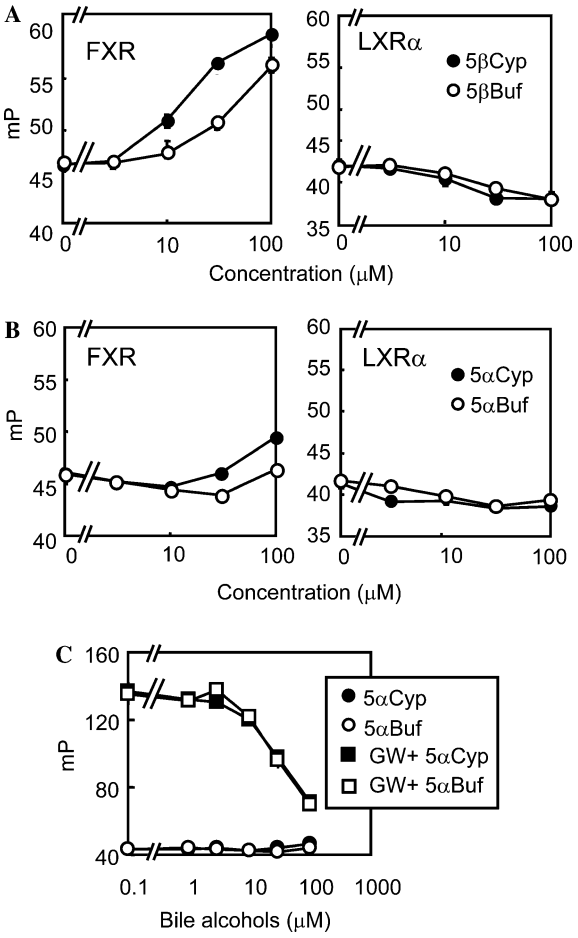

Fig. 3. 5β-Bile alcohols promote association between FXR and SRC-1 peptide in vitro, whereas 5α-bile alcohols function as antagonists, as determined by fluorescence polarization assay. (A,B) A fluorescence-tagged SRC-1 peptide (0.1 μM) was incubated with 1.5 μM GST-FXR or GST-LXRα in the presence of various concentrations of 5β-cyprinol (5β Cyp), 5β-bufol (5β Buf), 5α-cyprinol (5α Cyp), or 5α-bufol (5α Buf) indicated. Ligand-induced SRC-1 peptide association with the receptor was monitored by increases in millipolarization fluorescence units (mP). (C) Changes in fluorescence polarization caused by 3 μM GW4064 were measured in the presence of the concentrations of 5α-cyprinol (5α Cyp) or 5α-bufol (5α Buf) indicated. The values are means ± SD of three experiments. Some error bars are not visible within symbols.

(Fig. 2B). These two 5α-bile alcohols might have inhibited CDCA transport into the cells, and 5α-cyprinol has actually been shown to inhibit taurocholate uptake by *asbt*, the ileal conjugated bile acid transporter [25]. However, their inhibition of GW4064-elicited FXR transactivation and coactivator-association (Figs. 2C and 3C) indicated that they both competitively inhibit agonist-induced FXR activation. The results of the in vitro experiment (Fig. 3) also indicated that these bile alcohols directly activate FXR as ligands without being metabolized. These findings clearly show that 5α-cyprinol and 5α-bufol function as FXR antagonists.

The ability of 5β-cyprinol and 5β-bufol, and inability of their 5α-counterparts to promote coactivator-association to the receptor (Fig. 3) indicate that the A/B *cis* ring juncture (5β) is required for this process. However, the inhibition of GW4064-induced coactivator-association by the 5α-bile alcohols indicates that they are capable of binding to the receptor. Crystal structure studies have demonstrated interaction between the *cis*-oriented A ring of 5β-bile acids and residues on helix 12, corroborating the association between coactivator peptide and the receptor [10,11]. It is conceivable that the *trans*-oriented A ring in the 5α-bile alcohols destabilizes this agonist conformation of helix 12, thereby preventing coactivator-association.

Although 5α-cyprinol and 5α-bufol efficiently inhibited agonist-induced FXR transactivation and FXR-target gene expression, they had no effect in the absence of agonists. In a FXRE-dependent transactivation assay using HepG2 cells, luciferase activity of no-ligand control was very low, suggesting that the level of endogenous FXR ligands is negligible. Indeed, the bile acids produced in hepatic cells in vitro do not accumulate within the cells but are rapidly released to the medium [26], whereas in vivo the liver is constantly supplied with bile acids via the enterohepatic circulation. It is possible that the 5α-bile alcohols may inhibit FXR activation in the liver.

5α-Cyprinol and its sulfate are toxic and sometimes cause renal and hepatic failure after ingestion of goldfish or carp gallbladders [27,28]. A study has shown that 5α-cyprinol inhibits taurocholate uptake [25], but the mechanism of its toxicity is not well understood. By down-regulating BSEP and up-regulating CYP7A1 antagonizing FXR may lead to an increase in the intracellular level of toxic bile acids. In this study, we found that 5α-cyprinol and 5α-bufol antagonize CDCA-induced BSEP mRNA expression in HepG2 cells at concentrations that do not affect cell viability. Because BSEP plays the major role in bile acid excretion by the liver into the bile [29], and genetic defects in BSEP have been shown to cause progressive familial intrahepatic cholestasis [30], reduced BSEP expression may be involved in the mechanism of the toxicity.

The enhancement of CDCA-induced CYP7A1 mRNA repression by 5α-cyprinol and 5α-bufol was an unexpected finding (Fig. 4B). FXR antagonists should inhibit CDCA-elicited SHP induction and thereby diminish CYP7A1 repression. Exposure of cells to these 5α-bile alcohols alone also led to repression of CYP7A1 mRNA (Fig. 4A), although the SHP mRNA elevation was small or insignificant, suggesting a FXR/SHP-independent mechanism. It is noteworthy that ursodeoxycholic acid has been shown to repress CYP7A1 expression despite its negligible ability to activate FXR [31]. Studies have shown that CYP7A1 can be repressed by bile acids via redundant pathways, including repression through activation of the xenobiotic receptor pregnane X receptor (PXR) or activation of c-Jun N-terminal kinase mediated by TNFα or FXR-inducing FGF19 production [5,32–34]. A recent study has shown that 5α-cyprinol activates mouse PXR, but not human PXR [35], suggesting that the activation of a PXR-mediated pathway is unlikely in our experiments on HepG2 cells.

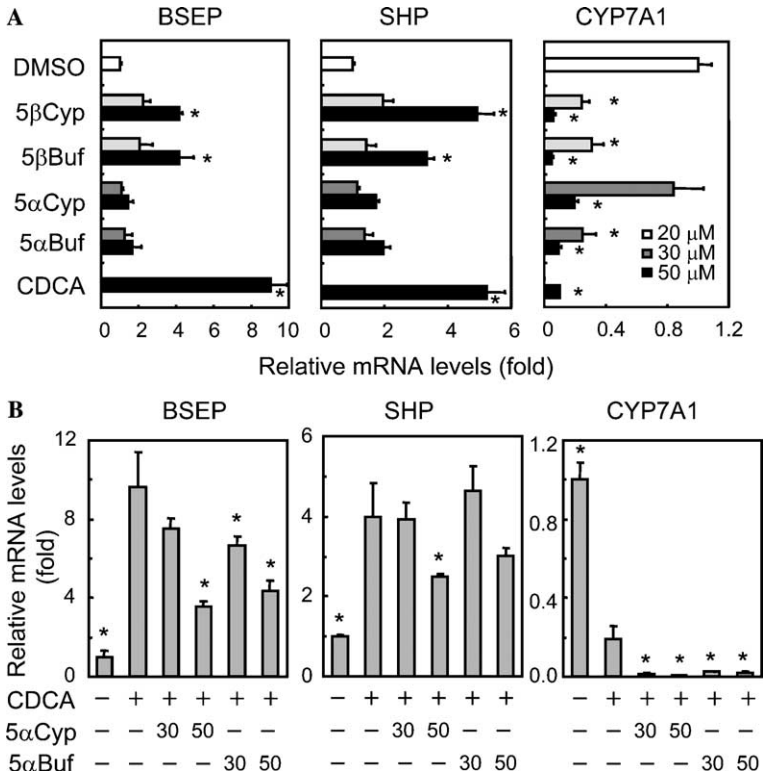

Fig. 4. Regulation of gene expression by various bile alcohols. HepG2 cells were treated for 20 h with vehicle (DMSO) alone, 50 μM CDCA, or the concentrations (20–50 μM) of bile alcohols indicated (A), or with 50 μM CDCA in the presence of the concentrations (μM) of 5α-cyprinol (5α Cyp) or 5α-bufol (5α Buf) indicated (B). Total RNA was isolated from the cells, and the levels of BSEP, SHP, and CYP7A1 mRNA were measured by real-time quantitative RT-PCR. Data were normalized to 18S rRNA levels. The values represent the means ± SD relative to vehicle-exposed cells (taken as 1) from three experiments. Statistically significant differences from respective controls (A) or the cells exposed to CDCA alone (B) are indicated by an asterisk ($*P < 0.01$).

A naturally produced FXR antagonist that lowers cholesterol has been identified [36], and an FXR agonist has been shown to prevent cholesterol gallstone formation [37]. Our findings may provide insights that will be useful for drug development.

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